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(54) Title: BIOCHEMICALLY ACTIVE AGENTS FOR CHEMICAL CATALYSIS AND CELL RECEPTOR ACTIVATION			
(57) Abstract <p>A biologically active composition made up of core particles or surfaces which are coated with a layer which is designed to allow attachment of biochemically reactive pairs (BRP's) without denaturing the BRP. BRPs which may be attached include ligand-receptor pairs, enzyme-substrate pairs, drug-receptor pairs, catalyst-reactant pairs, toxin-ligand pairs, absorbant-absorbate pairs and adsorbant-adsorbate pairs. Also disclosed are biologically active compositions made up of biodegradable core particles which are coated with a layer that is designed to allow attachment of biologically active agents without denaturing them. The compositions may further include an exterior targeting membrane which provides selective targeting to specific receptors. In addition, biologically active compositions for use in gene therapy and other transfection procedures are disclosed. The compositions are composed of nanocrystalline core particles which are coated with a layer that is designed to allow attachment of transfection agents (DNA/RNA segments or antisense fragments) without denaturing them. The composition may further include an exterior targeting membrane which provides selective targeting of the transfection agents to specific cell receptors.</p>			

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BIOCHEMICALLY ACTIVE AGENTS FOR CHEMICAL CATALYSIS AND CELL RECEPTOR ACTIVATION

BACKGROUND OF THE INVENTION

This is a continuation-in-part of co-pending application Serial No. 08/000,199 which was filed on January 4, 1993 which is a continuation-in-part of Serial No. 07/690,601 which was filed on April 24, 1991 which is a continuation-in-part of co-pending application Serial No. 07/542,255 which was filed on June 22, 1990.

1. Field of the Invention

The present invention relates generally to synthetic biologically active compositions which have a microparticulate (nanoparticulate) core. In one aspect, the present invention relates to biologically active compositions where transfecting DNA or RNA is attached to a microparticulate core and coated with a targeting membrane or ligand. These transfection nanoparticles are useful in delivering the transfecting DNA or RNA to target cells. In another aspect, the present invention relates to synthetic biochemically active agents which are useful for chemical catalyst and/or cell receptor activation. The present invention also relates to biologically active compositions where the microparticulate core is biodegradable.

2. Description of Related Art

The attachment of biologically active proteins, peptides or pharmacologic agents to various carrier particles has been an area of intense investigation. These conjugated biological systems offer the promise of reduced toxicity, increased efficacy and lowered cost of biologically active agents. As a result, many different carrier models are presently available.

(Varga, J.M., Asato, N., in Goldberg, E.P. (ed.): Polymers in Biology and Medicine. New York, Wiley, 2, 73-88 (1983). Ranney, D.F., Huffaker, H.H., in Juliano, R.L. (ed.): Biological Approaches to the Delivery of Drugs, *Ann. N. Y. Acad. Sci.*, 507, 104-119 (1987).) Nanocrystalline and micron
5 sized inorganic substrates are the most common carriers and proteins are the most commonly conjugated agents. For example, gold/protein (principally immunoglobulin) conjugates measuring as small as 5 nm have been used in immunological labeling applications in light, transmission electron and scanning electron microscopy as well as immunoblotting. (Faulk, W.,
10 Taylor, G., *Immunochemistry* 8, 1081-1083 (1971). Hainfeld, J.F., *Nature* 333, 281-282 (1988).)

Silanized iron oxide protein conjugates (again principally antibodies) generally measuring between 500 and 1500 nm have proven useful in various *in vitro* applications where paramagnetic properties can be used
15 advantageously. (Research Products Catalog, Advanced Magnetics, Inc., Cambridge, MA, 1988-1989.) Ugelstad and others have produced gamma iron oxides cores coated with a thin polystyrene shell. (Nustad, K., Johansen, L., Schmid, R., Ugelstad, J., Ellengsen, T., Berge, A.: Covalent coupling of proteins to monodisperse particles. Preparation of solid phase
20 second antibody. *Agents Actions* 1982; 9:207-212 (id. no. 60).) The resulting 4500 nm beads demonstrated both the adsorption capabilities of polystyrene latex beads as well as the relatively novel benefit of paramagnetism.

Carrier systems designed for *in vivo* applications have been fabricated from both inorganic and organic cores. For example, Davis and Illum
25 developed a 60 nm system comprised of polystyrene cores with the block copolymer poloxamer, polyoxyethylene and polyoxypropylene, outer coats that showed a remarkable ability to bypass rat liver and splenic macrophages. (Davis, S.S., Illum, L., *Biomaterials* 9, 111-115 (1988)). Drug
30 delivery with these particles has not yet been demonstrated. Ranney and

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Huffaker described an iron-oxide/albumin/drug system that yielded 350-1600 nm paramagnetic drug carriers. (Ranney, D.F., Huffaker, H.H., In, Juliano, R.L. (ed.): Biological approaches to the delivery of drugs, *Ann. N.Y. Acad. Sci.* **507**, 104-119 (1987).) Poznasky has developed an enzyme-albumin conjugate system that appears to decrease the sensitivity of the product to biodegradation while masking the apparent antigenicity of the native enzyme. (Poznasky, M.J.: Targeting enzyme albumin conjugates. Examining the magic bullet. In, Juliano, R.L. (ed.): Biological approaches to the delivery of drugs, *Annals New York Academy Sciences* 1987; 507-211:219.)

Shaw and others have prepared and characterized lipoprotein/drug complexes. (Shaw, J.M., Shaw, K.V., Yanovich, S., Iwanik, M., Futch, W.S., Rosowsky, A., Schook, L.B.: Delivery of lipophilic drugs using lipoproteins. In, Juliano, R.L.(ed.): Biological approaches to the delivery of drugs, *Annals New York Academy Sciences* 1987; 507:252-271.) Lipophilic drugs are relatively stable in these carriers and cell interactions do occur although little detail is known.

In any conjugated biological composition, it is important that the conformational integrity and biological activity of the adsorbed proteins or other biological agents be preserved without evoking an untoward immunological response. Spatial orientation and structural configuration are known to play a role in determining the biological activity of many peptides, proteins and pharmacological agents. Changes in the structural configuration of these compounds may result in partial or total loss of biological activity. Changes in configuration may be caused by changing the environment surrounding the biologically active compound or agent. For example, pharmacologic agents which exhibit *in vitro* activity may not exhibit *in vivo* activity owing to the loss of the molecular configuration formerly determined in part by the *in vitro* environment. Further, the size and associated ability of the carrier particle to minimize phagocytic trapping

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is a primary concern when the composition is to be used *in vivo*. All of these factors must be taken into account when preparing a carrier particle.

Biochemical phenomena consist of binary interactions between pairs of molecules. Common names for such biochemically reactive pairs ("BRP's") include but are not limited to immunological pairs, ligand-receptor pairs, enzyme-substrate pairs, drug-receptor pairs, catalyst-reactant pairs, catalyst-substrate pairs, absorbate-absorbent pairs, adsorbate-adsorbent pairs, and toxin-ligant pairs. On a molecular level, nearly all biochemical phenomena between such pairs involve the spatial recognition of one molecule by another, and such recognition serves as the means by which energy and information are transmitted, products are generated, responses are initiated and complex biological structures are built.

The process of spatial recognition implies of both regioselective and stereoselective interactions among BRP's. One member of a BRP, constrained by fundamental biophysical laws, may interact with the other member of a BRP if and only if both members are physically conformed within some bounded set of possible spatial arrangements and if both members have their respective interactive regions unencumbered. The environment within which BRP's interact affect greatly the process of spatial recognition. Environments that constrain spatial mobility or encumber molecular regions may, depending on the degree of constraint and the resulting spatial conformation, either promote or inhibit BRP interactions.

An example of the former is surface activation of synthetic chemical reactions in a process known as "solid phase synthesis." Solid phases, either as solid glassy polymers, crystalline materials, or complex macromolecular polymers have been features of synthetic biochemistry since the early 1960's. Their use was advanced largely by Merrifield for facilitating peptide synthesis and for which he received the Nobel Prize for Chemistry in 1984. They became widely popular because the solid-phase method

offered simplicity, speed, avoidance of intermediate isolation, and automation. The principal limitation in the widespread use of solid phases has been the empirical observation that only a few surfaces have been effective BRP interaction promoters.

5 Drug delivery systems which are used for *in vivo* delivery of biologically active agents, tend to be very complex. Most have some advantages that give them promise and possibility for further development. Most are also riddled with problems ranging from low drug load to *in vivo* instability. Drug delivery systems which are able to deliver a moderate
10 dose of therapeutic agent to a specific target cell are highly desirable. This type of delivery system should provide a binding of the carrier to the cell via a receptor and a mechanism of internalization where the drug is liberated to the intracellular space from the carrier. The carrier should also be biodegradable and not induce any type of immune reaction or response.
15 The delivery system should be specific, with high efficacy and low non-targeted toxicity. It should be convenient to administer, manufacture, and be economically viable for both the patient and the manufacturer.

Although numerous different carrier particles have been developed, there is a continuing need to provide carrier particles for both *in vivo* and
20 *in vitro* application wherein a biologically active peptide, protein or pharmacological agent can be attached to the particles in a manner which promotes stabilization of the biologically active compound in its active configuration. With respect to chemical catalysts and cell receptor activation, it would be desirable to develop synthetic surfaces to which
25 individual catalysts may be anchored without destroying their catalytic activity. Such surfaces should also be useful for immobilizing biochemically reactive pairs (BRP's), such as catalyst substrate pairs, without reducing the ability of the BRP's to promote biochemical reactions.

To date, gene therapy in humans has been limited to *ex-vivo* protocols
30 in which tissues are transfected in the culture dish and placed back in

the body. *In vivo* work is still in pre-clinical development and has been confined to animal models due to a range of safety and efficacy issues. Such concerns arise primarily from the use of viral vectors to effect the gene transfer. Retroviral transfection has generated a lot of interest since it can stably transfect nearly 100% of targeted cells *ex-vivo*. Production of transfecting, replication defective retroviruses, proceeds through packaging cell lines which in principle are unable to produce wild type virus. However, low titers of "wild type" (replication competent) virus have been observed in these systems. In one such protocol utilizing primates, outbreaks of lymphoma were linked to the detection of wild type retrovirus from a packaging system. Besides potential pathogenicity, maintaining useful transfecting titers of these vectors can be difficult. They are hard to purify and concentrate since the envelopes (membranes) tend to be extremely labile. Alternatively, adenoviral vectors have been found to be considerably more stable. Moreover, these viruses are capable of transfecting quiescent tissue and producing large amounts of gene products. Unfortunately, gene expression is often transient because the viral genome often remains extra-chromosomal. Direct clinical application is also problematic, since replication of the vector can result in aberrant host protein synthesis leading to deleterious effects ranging from oncogenesis to cellular toxicity.

Given the practical concerns of *in vivo* viral transfection, nonviral methods are also being developed. At present, most efforts are centered on receptor mediated transfer because such methods can provide targeted delivery of DNA (and RNA) *in vivo*. Receptor-mediated systems employ ligand DNA (and RNA) complexes which can be recognized by cell receptors on the cell surface. Internalization of the complex occurs via the formation of endocytotic vesicles which allow for transport into the cytoplasm. Problems arise, however, when the endosomes fuse with lysosomes which causes the contents to be destroyed. In turn, the trans-

fection rate for these complexes remain below clinical efficacy. Some investigators have used fusogenic peptides of Influenza Hemmoglutin A to disrupt endosome formation which has led to higher transfection rates. Nonetheless, the data on *in vivo* expression suggests that this method may only permit transient expression of genes.

Besides ligand DNA (and RNA) complexes, lipofection techniques have also been tried with varying success. Liposomes are specially susceptible to uptake by the filter organs and in the peripheral tissues by macrophages which limits their transfection efficiency and specificity *in vivo*.

In view of the above, it would be desirable to provide compositions which can be used to transfect cells with DNA or RNA in both *in vivo* or *in vitro* environments.

SUMMARY OF THE INVENTION

In accordance with the present invention, members of a BRP such as biologically active peptides, proteins or pharmacological agents are attached to a core particle to provide a wide variety of biologically active compositions. The invention is based on the discovery that the surface of ultrafine particles (nanocrystalline particles) can be modified with a surface coating to allow attachment of biologically active moieties to produce compositions wherein the naturally occurring structural environment of the moiety is mimicked sufficiently so that biological activity is preserved. The coating which provides for the attachment of biologically active moieties to nanocrystalline particles in accordance with the present invention can be composed of a basic or modified sugar or oligonucleotide. Coating nanocrystalline particles with a basic sugar or oligonucleotide produces changes in the surface energy and other surface characteristics which make the particles well suited for attachment of biologically active moieties or other members of a BRP.

In accordance with the present invention, ultrafine core particles having diameters of less than about 1000 nanometers are used to anchor enzymes or other catalytic particles without denaturing the catalyst. Surface coating of the core particles provides an anchoring surface which prevents substantial alteration of the catalysts which might otherwise occur when the catalysts are attached directly to the particle surface. The coated particles are also useful for anchoring catalyst-substrate (enzyme-substrate) pairs or other bioreactive pairs without destroying the catalytic activity of the BRP's.

The present invention also involves the attachment of BRP's to macroscopic surfaces, such as films or solid surfaces. Anchoring of BRP's to these type of large surface area systems are useful where chemical catalysis or cell receptor activation is not dependent upon or does not require the microgeometry provided by nanocrystalline core anchoring particles.

As a further aspect of the present invention, biologically active peptides, proteins or pharmacological agents are attached to a biodegradable core particle to provide a wide variety of biologically active compositions. The invention is based in part on the discovery that the surface of ultrafine particles (nanocrystalline particles) can be modified with a surface coating to allow attachment of biologically active moieties to produce compositions wherein the naturally occurring structural environment of the moiety is mimicked sufficiently so that biological activity is preserved. The core particle, with the surface coating and attached moiety, is further coated with a targeting agent, such as a biologically active ligand or phospholipid membrane complex.

The invention is also based in part on the discovery that biodegradable core particles may be assembled with biologically active or pharmaceutical agents to form a biologically active core which is further treated

with a targeting ligand or membrane complex to provide selective targeting of the biochemically active core *in vivo*.

As a feature of the present invention, the nanocrystalline core is composed of brushite which is composed of crystalline calcium phosphate (brushite). This material is biodegradable, inexpensive and is found in human beings as a substrate of bone synthesis. The brushite particles may be synthesized at a nanomeric size (between approximately 5 nm and 150 nm). This small size allows the drug delivery construct to be small enough to avoid uptake by the Reticulo-Endothelial System (RES) of the body and deliver the drug or biologically active agent *in vivo* without non-specific toxicity or loss of drug to macrophages.

The brushite core may be co-crystallized with a drug or agent for sustained release and protection of the drug from the physiological environment. Conversely, the brushite core may be coated, as described above with a sugar, such as cellobiose or pyridoxal-phosphate to improve its surface attachment characteristics. The sugar coat allows for the binding of drugs and other biological agents in their active or inactive forms, without alteration. The sugar helps to maintain the conformation of the drug or agent that is adsorbed to its surface. This core construct is capable of carrying a moderate drug or agent load, depending upon the size of the biological agent.

As a feature of the present invention, the brushite based particle is targeted to a specific tissue or cell type. In order to achieve this targeting, the construct has a targeting ligand or a primed phospholipid membrane tightly adsorbed to its surface. The membrane may contain proteins, receptors and carbohydrates which provide targeting of the vehicle. The membrane also serves to further maintain the stability of the biological agent and the integrity of the construct. This membrane may be derived from cell membranes, viral envelopes (see U.S. patent 5,178,882), or other specifically engineered or synthesized membranes. Due to the very small

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size of the biodegradable core particle delivery system, multiple layers of membranes may be adsorbed to the core particle to increase the efficiency of targeting.

As one aspect of the present invention, biodegradable nanocrystal-
line particles are used to prepare a decoy virus wherein the DNA or RNA
core of the virus is replaced by the microparticle. The microparticle is
chosen to be approximately the same size as the viral core so that the
conformation of the surrounding protein coat accurately mimics the native
virus. The resulting viral decoy is incapable of infectious behavior while at
the same time being fully capable of effecting an immune response and
otherwise being antigenically bioreactive.

The biologically active microparticles in accordance with the present
invention have wide-ranging use depending upon the type of biologically
active compound which is attached to the biodegradable microparticle core.
When viral protein from HIV is attached to the microparticle core, the result
is a decoy virus which may be used as an AIDS vaccine, diagnostic tool or
antigenic reagent for raising antibodies. Non-viral protein or antigen
coatings may be selected and structured for use in raising specific anti-
bodies or as a diagnostic tool. Further, the microparticles can function as
a pharmacological agent when compounds having pharmacological activity
are attached to the biodegradable core particle.

In accordance with the present invention, biodegradable core parti-
cles having diameters of less than about 1000 nanometers are used to
anchor enzymes or other catalytic particles without denaturing the catalyst.
Surface coating of the core particles provides an anchoring surface which
prevents substantial alteration of the catalysts which might otherwise
occur when the catalysts are attached directly to the particle surface. The
coated particles are also useful for anchoring catalyst-substrate (enzyme-
substrate) pairs or other bioreactive pairs (BRP's) without destroying the
catalytic activity of the BRP's.

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As an additional aspect of the present invention, transfecting DNA or RNA is attached to a nanocrystalline core particle and coated with a targeting ligand or membrane to provide a viral transfection system which may be used in gene therapy. The invention is based in part on the discovery that the surface of ultrafine particles (nanocrystalline particles) can be modified with a surface coating to allow attachment of transfecting DNA or RNA to produce compositions wherein the naturally occurring structural environment of the DNA or RNA is mimicked sufficiently so that biological activity is preserved. The core particle, with the surface coating and attached transfecting DNA or RNA, is further coated with a targeting agent, such as ligand or phospholipid membrane complex to provide targeting of the DNA or RNA to particular cell receptors.

As a feature of this aspect of the present invention, the nanocrystalline core may be composed of brushite. This material is biodegradable, inexpensive and is found in human beings as a substrate of bone synthesis. The brushite particles may be synthesized at a nanomeric size (between approximately 5 nm and 150 nm). This small size allows the DNA/RNA delivery construct to be small enough to avoid uptake by the Reticulo-Endothelial System (RES) of the body and deliver the DNA/RNA to cells *in vivo* without non-specific toxicity or loss of drug to macrophages.

The DNA/RNA-particle construct is targeted to a specific tissue or cell type. In order to achieve this targeting, the construct has a targeting ligand or a primed phospholipid membrane tightly adsorbed to its surface. The membrane may contain proteins, receptors and carbohydrates which provide targeting of the vehicle. The membrane also serves to further maintain the stability of the transfecting DNA or RNA and the integrity of the construct. This membrane may be derived from cell membranes, viral envelopes (see U.S. patent 5,178,882), or other specifically engineered or synthesized membranes. Due to the very small size of the biodegradable core particle delivery system, multiple layers of membranes may be

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adsorbed to the core particle to increase the efficiency of targeting. The DNA/RNA transfecting microparticles in accordance with the present invention have wide-ranging use depending upon the particular DNA or RNA which is attached to the biodegradable microparticle core.

5 The above-discussed and many other features and attendant advantages of the present invention will become better understood by reference to the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention has wide application to immunologic procedures and methods wherein antigenic material, biologically reactive pairs (BRP's) or other biologically active moieties are utilized. These areas of application include vaccination agents, antigen agents used to raise antibodies for subsequent diagnostic uses and antigenic compounds used as
15 diagnostic tools. The composition of the invention can also be used in a wide variety of other applications where there is a need to target pharmaceuticals or other biologically active agents to particular cell types in both *in vivo* and *in vitro* environments.

20 The compositions of the present invention include biodegradable nanocrystalline core particles (diameters of less than 1000 nm) which are coated with a surface energy modifying layer that promotes bonding of proteins, peptides or pharmaceutical agents to the particles. The coating modifies the surface energy of the nanocrystalline core particles so that a wide variety of immunogenic proteins, peptides and pharmaceutical agents
25 may be attached to the core particle without significant loss of antigenic activity or denaturation. The result is a biologically active composition which includes a biologically inert core. The end use for the compositions of the present invention will depend upon the particular protein, peptide or pharmacological agent (including gene therapy) which is attached to the
30 coated core particle. For example, proteins or peptides having antigenic

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activity may be attached to provide compositions useful as immunodiagnostic tools. Viral fragments or protein coatings having immunogenic activity may be attached to provide a vaccine. Also, pharmacological agents may be attached to provide compositions which are useful in treating diseases. Also, gene segments or antisense fragments may be attached which are useful in treating diseases.

Examples of individual catalysts which may be attached to the coated core particles include tissue plasminogen activator (whole and partial domains), trypsin inhibitor, cytochromes, Ferredoxin, phosphotransferase, acyltransferase, papain, Lys C, Arg C, Trypsin, Coagulation factor V, Xlla, Xla, VIIa, Complement factor C3, C3b and properdin.

Bioreactive pairs (BRP's), such as enzyme-substrate pairs, may also be attached to the coated core particles. Exemplary enzyme-substrate pairs include: lysozyme-chitin pairs, where the lysozyme catalyzes the hydrolysis of NAM and NAG glycosidic bonds; ribonuclease-RNA pairs where the ribonuclease catalyzes the hydrolysis of RNA; carboxypeptidase A — carboxyl terminal polypeptide pairs where the enzyme catalyzes the hydrolysis of the carboxyl-terminal peptide bond in the polypeptide chain; serine, zinc, thiol and carboxyl proteases-protein pairs where the protease catalyze the degradation of the protein; NADH-Q reductase — NADH pairs where the reductase catalyzes the oxidation of NADH and the reduction of Q; glutathione seductase-glutathione pairs; acetylcholinesterase-acetylcholine pairs; Lys C; Arg C; acyl and acyltransferase; aspartate and aspartate carbamoyltransferase; elastase; and the cytochromes.

Other biochemically reactive pairs (BRP) which can be immobilized on to the coated solid surfaces in accordance with the present invention include members of immunological pairs, ligand-receptor pairs, drug-receptor pairs, catalyst reactant pairs, catalyst-substrate pairs, absorbate-absorbent pairs, adsorbate-adsorbent pairs, and toxin-ligand pairs. Such members include but are not limited to:

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- immunological pair members such as IgG, IgM, IgA, IgE and IgD, whole or in part as in Fc or Fab fractions, polyclonal or monoclonal, with recognition sites for epitopes on cells (cell surface antigens) such as CD1, CD3, CD4, CD8, CD11, CD25, CD68; viral epitopes such as EBVgp350, HIVp24, HIVgp120, MS virus coat protein (bacteriophage) other viral antigens, bacterial antigens, fungal antigens, and known viruses, fungi, bacteria, prions and protozoa.
5
- ligand-receptor pair members such as lectins and lectin binding sites such as FVIII receptor; HDL and HDL receptor cellular receptor site; hormones such as estrogen and estrogen receptor sites; antibiotics; ribosomal proteins; FK506 and FK506 binding protein; ricin and cell target; phosphotyrosine recognition domain SH2 (RSV) and phosphotyrosine; and (oligo)nucleopeptides and their corresponding antisense nucleopeptide.
10
15
- drug-receptor pair members such as epinephrine and adrenergic receptors, methadone and opiate receptors, DNA chelating agents such as adriamycin, etc.
20
- catalyst-reactant pair members such as iron and superoxide, rhodopsin kinase and rhodopsin, hydrogen peroxide and luminol, horseradish peroxidase and hydrogen peroxide.
- adsorbent-adsorbate pair members, such as trypsin-trypsin inhibitor, biotin-biotin repressor (E. coli) and subtilisin and subtilisin inhibitor.
25
- toxin-ligand pair members such as strychnine and the glycine receptor, hemoglobin and carbon monoxide, and organophosphate compounds (sarin, tabun, parathion, dimefox, malathion, diazinon)
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and acetylcholinesterase; muscarinic receptor and neurotoxins (Neurotoxin I from *S. helianthus*' scorpion neurotoxin); verotoxin and colonic mucosal epithelial receptor; enterotoxin and colonic mucosal epithelial receptor.

5

One or both of the members of the BRP may be initially bound to the modified surface. In general, the enzyme or catalyst will be bound first and substrate or reactant bound later during actual interaction between the enzyme and substrate or catalyst and reactant.

10

For preparing decoy viruses for use as vaccines, particles having diameters of between about 10 to 200 nanometers are preferred since particles within this size range more closely mimic the diameter of DNA and RNA cores typically found in viruses.

15

The core particles used to anchor catalysts or BRP's can have a much broader size range than the particles used in decoy viruses. The particle size should be chosen to maximize the catalytic or enzymatic reaction of the BRP. Preferred particle sizes are in the range of 50 to 150 nm. If desired, the BRP may be attached to a macroscopic surface, such as a film or solid substrate surface.

20

The core particles or other surfaces may be made from a wide variety of inorganic materials including metals or ceramics. Preferred metals and alloys include beryllium, silicon, gallium, copper, gold, titanium, nickel, aluminum, silver, iron, steels, cobalt-chrome alloys, and titanium alloys. Preferred ceramic materials include calcium-phosphate, alumina, silica, and zirconia. The core particles may be made from organic materials including carbon (diamond). Preferred polymers include polystyrene, silicone rubber, polycarbonate, polyurethanes, polypropylenes, polymethylmethacrylate, polyvinylchloride, polyesters, polyethers, and polyethylene.

25

30

The core particles may be made from a variety of biodegradable materials including polymers or ceramics. Preferred ceramic materials

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include brushite composed of calcium phosphate dihydrate, and tricalcium phosphate and iron oxide. Preferred biodegradable polymers include polylactide, polygalactide and polylysine. Particles made from brushite are particularly preferred.

5 Particles made from the above materials having diameters less than 1000 nanometers are available commercially or they may be produced from progressive nucleation in solution (colloid reaction), or various physical and chemical vapor deposition processes, such as sputter deposition (Hayashi, C., *J. Vac. Sci. Technol.* A5 (4), Jul/Aug. 1987, pgs. 1375-1384; Hayashi, C., *Physics Today*, Dec. 1987, pgs. 44-60; MRS Bulletin, Jan 1990, pgs. 10 16-47). Tin oxide having a dispersed (in H₂O) aggregate particle size of about 140 nanometers is available commercially from Vacuum Metallurgical Co. (Japan). Other commercially available particles having the desired composition and size range are available from Advanced Refractory 15 Technologies, Inc. (Buffalo, N.Y.).

Plasma-assisted chemical vapor deposition (PACVD) is one of a number of techniques that may be used to prepare suitable microparticles. PACVD functions in relatively high atmospheric pressures (on the order of one torr and greater) and is useful in generating particles having diameters 20 of up to 1000 nanometers. For example, aluminum nitride particles having diameters of less than 1000 nanometer can be synthesized by PACVD using Al (CH₃)₃ and NH₃ as reactants. The PACVD system typically includes a horizontally mounted quartz tube with associated pumping and gas feed systems. A susceptor is located at the center of the quartz tube and heated using a 60 KHz radio frequency source. The synthesized alumi- 25 num nitride particles are collected on the walls of the quartz tube. Nitrogen gas is used as the carrier of the Al (CH₃)₃. The ratio of Al (CH₃)₃: NH₃ in the reaction chamber is controlled by varying the flow rates of the N₂/Al(CH₃)₃ and NH₃ gas into the chamber. A constant pressure in the 30 reaction chamber of 10 torr is generally maintained to provide deposition

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and formation of the ultrafine nanocrystalline aluminum nitride particles. PACVD may be used to prepare a variety of other suitable nanocrystalline particles.

5 The core particles or other surface are coated with a substance that provides a threshold surface energy to the particle or other surface which is sufficient to cause binding to occur without that binding being so tight as to denature biologically relevant sites. For particles, coating is preferably accomplished by suspending the particles in a solution containing the dispersed surface modifying agent. It is necessary that the coating make
10 the surface of the particle more amenable to protein or peptide attachment. For surfaces, the coating may be applied to a meticulously clean area located on the surface.

Suitable coating substances in accordance with the present invention include carbohydrates, carbohydrate derivatives, and other macromolecules
15 with carbohydrate-like components characterized by the abundance of -OH (hydroxyl) side groups. The coatings may include but are not limited to:

- short chain carbohydrates including glucose, sucrose, cellobiose, nystose, triose, dextrose, trehalose, glucose, lactose, maltose, etc.
- 20 • hydroxyl rich weak acids such as citrate, fumarate, succinate, isocitrate, oxaloacetate, malate, etc.
- nucleotide-like molecules with pendant carbohydrate or phosphate groups such as pyridoxyl-5-pyrophosphate, thiamine pyrophosphate,
25 uridine-diphosphate-glucose, glucose-1-phosphate, adenosine, nicotinamide-adenine-diphosphate, etc.
- derivatives of carbohydrates such as nitrocellulose

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- complex polymeric carbohydrates and derivatives such as dextran, glycogen, etc.

5 Preferred coating materials include cellobiose, sucrose, pyridoxyl-5-phosphate and citrate.

An exemplary preferred method for binding the stabilizing coat to the solid phase followed by a member of a BRP consists of:

1. obtaining a meticulously clean surface on the solid to be coated;
- 10 2. immersion of the meticulously clean surface to be coated in an aqueous solution of the coating material followed by;
3. lyophilization of the aqueous solvent/dispersant from the surface of the solid;
- 15 4. immersion of the coated solid surface in an aqueous solution/dispersion containing (a) member(s) of a biochemically reactive pair (BRP); and
- 20 5. removal of the aqueous solvent yielding a solid coated with a molecular stabilizing film to which is bound (a) BRP member(s).

As used in Step 1 above, the term "meticulously clean surface" means a surface of a material that has been cleansed of all matter that is not intrinsic to the material comprising the bulk of the solid to be coated. If a solid of some composition A is already coated with a second solid of some composition B, and it is desired that the molecular stabilizing film be applied to the surface of composition B, then the term refers to surface B and matter that is not intrinsic to the material comprising the bulk of B. Such techniques include the individual or combined application of acids,

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bases, sonic energy, plasma glow discharge processes, and even mechanical cleansing to preformed surfaces.

Immersion, as used in Step 2 above, means the application of a contiguous layer of the solution to the surface undergoing coating. Such techniques as spraying, dipping, mechanical painting, or other means of transfer are intended insofar as they yield a contiguous layer comprised exclusively of the solvent and coating macromolecule on the surface to be coated.

Lyophilization, as used in Step 3 above, means the removal of the aqueous phase from the surface film by a reduction in the ambient gas partial pressure. Both the application of heat and the removal of heat to cool the solid and the newly forming surface film may be modifications of the lyophilization process.

Immersion, as used in Step 4 above, means the application of a contiguous layer of a solution containing (a) member(s) of a BRP to the already modified surface of the coated solid. Such techniques as spraying, dipping, mechanical painting, or other means of transfer are implied insofar as they yield a contiguous layer comprised exclusively of the solvent and BRP members on the already modified surface consisting of a molecular stabilizing film to be coated.

Removal, as used in Step 5 above, means the removal of the aqueous phase from the surface film by (a) a reduction in the ambient gas partial pressure (lyophilization) or (b) dialysis/ultrafiltration. Both the application of heat and the removal of heat to cool the solid and the newly forming surface film may be modifications of the lyophilization process.

With respect to particles, the particles are suspended in a coating solution. The coating solution into which the core particles are suspended contains, for example, from 1 to 30 weight/volume percent of the coating material. The solute is preferably double distilled water (ddH₂O). The amount of core particles suspended within the coating solution will vary

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depending upon the type of particle and its size. Typically, suspensions containing from 0.1 to 10 weight/volume percent are suitable. Suspensions of approximately 1 weight/volume percent of particles are preferred.

5 The core particles are maintained in dispersion in the coating solution for a sufficient time to provide uniform coating of the particles. Sonication is the preferred method for maintaining the dispersion. Dispersion times ranging from 30 minutes to a few hours at room temperature are usually sufficient to provide a suitable coating to the particles. The thickness of the coating is preferably less than 5 nanometers. Thicknesses of the
10 coating may vary provided that the final core particles include a uniform coating over substantially all of the particle surface.

The particles are separated from the suspension after coating and may be stored for future use or redispersed in a solution containing the protein or peptide to be attached to the particles. Alternatively, the coated
15 particles may be left in the suspension for further treatment involving attachment of the desired protein or peptide.

The term "biodegradable" as used herein means any core particle which decomposes or otherwise disintegrates after prolonged exposure to a mammalian *in vivo* environment. To be biodegradable, the core particle
20 should be substantially disintegrated within a few weeks after introduction into the body.

The protein or peptide which is applied to the coated particles may be selected from a wide variety of proteins or peptides. Those having antigenic properties are preferred when a vaccine is required. The protein
25 can be the viral protein coat from a selected virus or immunogenic portion thereof. The viral protein coat is isolated according to known separation procedures for isolating and separating viral proteins. The viral coating is the preferred protein because the viral coating is where the antigenic activity of viruses is known to be located. Typically, the virus is digested
30 or solubilized to form a mixture of viral proteins. The viral proteins are then

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separated by liquid chromatography or other conventional process into the various protein particle fractions and dialyzed to remove impurities.

Suitable viruses from which viral protein particles can be separated and isolated include Epstein-Barr virus, human immunodeficiency virus (HIV), human papilloma virus, herpes simplex virus and pox-virus. Preparations of a wide variety of antigenic protein materials may also be purchased commercially from supply houses such as Microgene Systems, Inc. (400 Frontage Road, West Haven, Connecticut 06516), Amgen Corporation (1900 Oak Terrace Lane, Thousand Oaks, California 91320-1789) and Cetus Corporation (1400 53rd Street, Emeryville, California 94608 and Advanced Biotechnology, Inc. (Columbia, Maryland). Synthetic peptides and/or proteins which correspond to naturally occurring viral particles may also be utilized.

With respect to HIV, any of the viral fragments which are known to elicit an immune response can be used. Suitable viral fragments include gp120, gp160, gp41, and core proteins (p24). Any of the known techniques for preparing HIV fragments may be used including recombinant methods.

Other biologically active proteins and peptides that can be attached include enzymes, hormones, transport proteins and protective proteins. Human serum transferrin, plasminogen activator and coagulation factors, in addition to the pharmacologic agents amphotericin, taxol and insulin, are examples.

The procedure for attaching the antigens or other protein to the coating on the core particles involves suspending the coated core particles in an aqueous solution containing the antigen. The presence in the solution of materials that may preferentially attach to the particle surface is often not advantageous. For example, the dispersion agents present in the solution may create an undesirable coating on the suspended particles prior to protein attachment. Water miscible solvents such as methanol or ethanol

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may be used. The aqueous solution of coated microparticles can be agitated sufficiently to provide a uniform suspension of the particles. Typically, the amount of particles in solution will be between about 0.5 mg per milliliter of solution and 5 mg per milliliter of solution. Sonication is a preferred method for providing a uniform suspension of the coated particles in solution.

The suspension of coated particles and antigens must be within certain parameters for protein attachment and self assembly to occur. The temperature of the particle solution should be between 1°C to 45°C. Certain proteins and pharmaceutical agents may be bound to the coated particles in distilled water. Salts may be added to the solution for reactions between coated particles and proteins and other pharmaceutical agents which are unstable or will not disperse readily in distilled water. In general, the salt solutions should be formulated so that the ionic balance (in mM) does not exceed: K=300-500; Na=30-70; Cl=40-150; Ca=0.0003-0.001; and Mg=0.0003-0.001. The oxygen tension of the solution is, advantageously, less than 10% in a solution sparged initially by helium and then gassed with helium, nitrogen and carbon dioxide. The pH of the solution is, advantageously, slightly acidic (relative to blood), with a value, preferably, of between 6.8 to 7.2. An exemplary solution for dispersion of the coated microparticles and for protein attachment is an aqueous solution containing: 0.0360 milligrams MgSO_4 per liter, 0.0609 milligrams $\text{MgCl}_{2.6}\text{H}_2\text{O}$, 0.0441 milligram $\text{CaCl}_{2.2}\text{H}_2\text{O}$, 22.823 grams K_2HPO_4 , 13.609 grams KH_2PO_4 , 7.455 grams KCl, and 4.101 gram sodium acetate. The pH of this solution is adjusted to 6.8.

The coated particle cores with the attached protein can be separated from the ionic growth medium and stored for further use. The coated particles may be stored by any of the conventional methods typically used for storing antigenic compounds or antibodies. For example, the coated particles may be freeze dried (lyophilized) or stored as a suspension in a

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compatible solution. When used as a vaccine, the particles coated with a viral protein coat are injected or otherwise administered to the individual according to conventional procedures. Any pharmaceutically acceptable carrier solution or other compound may be used in administering the coated particles to the individual. When used for diagnostic purposes *in vitro*, the protein coated particles are suspended in solution and used in the same manner as other antigenic compounds. The same is true for use of the protein coated particles for raising antibodies. The same protocol and procedures well known for using antigens to produce antibodies may be used wherein the protein coated particles of the present invention are substituted for normally used antigenic compounds.

When targeting of the coated particle and attached biologically active agent is desired, the particles may be coated with a target ligand or a phospholipid membrane complex which is reactive with receptors on particular cells. Exemplary target ligands include HIV coat proteins (gp160, 41, 120) corona virus coat proteins, EBV coat proteins (gp350). Any membrane bound ligand/receptor may be used. These ligands are attached to the particle complex in the same manner as attachment of the biologically active agents discussed above.

The lipids used to coat the biodegradable nanocrystalline particle and bound agent may also be the same lipids commonly used to form liposomes. Suitable lipids include phospholipids such as phosphatidylcholine, cholesterol and phosphatidylserine. The lipids may also be derived directly from natural sources. Such lipids include viral membranes and other lipid bound biochemically reactive pairs. The lipid layer is applied to the nanocrystalline core particle and bound to a biologically active agent in the same manner as the surrogate red blood cells described in United States Patent No. 5,306,508.

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The core particle and bound agent do not need to be totally covered with a lipid layer. Preferably, the amount of lipid used to coat the particle will be sufficient to coat the entire particle.

5 In certain situations, it is desirable to attach the biochemically active agent directly to the biodegradable core particles. This direct attachment is preferably accomplished by co-crystallizing the biodegradable core particle with the biochemically active agent. For example insulin, taxol or (DNA, RNA) gene fragments may be co-crystallized with brushite to form drug loaded particles which provide sustained release of the drug while
10 providing protection of the drug *in vivo*. This direct attachment reduces biological activity.

The present invention also has wide application to procedures and methods wherein DNA or RNA are delivered, i.e. transfected, to cells *in vivo* or *in vitro*. These areas of application include gene therapy. The
15 compositions of the present invention can be used in a wide variety of other applications where there is a need to target DNA or RNA to particular cell types in both *in vivo* and *in vitro* environments. The invention may also be used to target other transfection agents, such as antisense fragments. The term "transfection agent" as used herein is intended to mean
20 DNA or RNA segments or antisense fragments which are capable of transfection into a cell. Exemplary transfection agents include sense DNA, sense RNA, antisense RNA, and antisense DNA.

The compositions of the present invention include nanocrystalline core particles (diameters of less than 1000 nm) which are coated with a
25 surface energy modifying layer that promotes bonding of proteins, peptides or pharmaceutical agents to the particles. The coating modifies the surface energy of the nanocrystalline core particles so that DNA and RNA segments may be attached to the core particle without significant loss of activity or denaturization. The result is a biochemically active composition
30 which includes a biochemically inert core. The end use for the composi-

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tions of the present invention will depend upon the particular transfection agent which is attached to the coated core particle. For example, DNA segments, such as human low density lipid receptor, are used in gene therapy. RNA segments, such as antisense and sense mRNA, are used in the transfection procedure. Antisense fragments, such as HIV reverse transcriptase are used in the transfection particles. Preferred particle sizes are on the order of 10 nm to 150 nm.

The core particles and coatings are the same as described above. The particles are separated from the suspension after coating and may be stored for future use or redispersed in a solution containing the DNA or RNA to be attached to the particles. Alternatively, the coated particles may be left in the suspension for further treatment involving attachment of the desired DNA or RNA.

The core particle may be made from a biodegradable ceramic or polymer. The term "biodegradable" as used herein means any core particle which decomposes or otherwise disintegrates after prolonged exposure to a mammalian *in vivo* environment. To be biodegradable, the core particle should be substantially disintegrated within a few weeks after introduction into the body. Brushite is a preferred biodegradable core particle material.

The DNA or RNA which is applied to the coated particles may be selected from a wide variety of DNA or RNA segments which are used to transfect cells during gene therapy. Antisense fragments may also be used.

Exemplary transfection gene segments include human low density lipid receptor CDNA, human adenosine deaminase CDNA, human dystrophin CDNA, and antisense HIV reversed DNA — all subcloned within appropriate expression vectors. The DNA or RNA transfection segments may be prepared according to known procedures such as the procedure described in Manniatis, T., Fritsch, E.F., and Sambrook, S., Molecular Cloning, Cold Spring Laboratory Press, N.Y., 1.0-19.0 (1989). Gene

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segments are also available commercially from a number of different suppliers.

The procedure for attaching gene segments or antisense fragments to the coating on the core particles involves suspending the coated core particles in an aqueous solution containing the gene segments. The presence in the solution of materials that may preferentially attach to the particle surface is often not advantageous. For example, the dispersion agents present in the solution may create an undesirable coating on the suspended particles prior to protein attachment. Water miscible solvents such as methanol or ethanol may be used. The aqueous solution of coated microparticles can be agitated sufficiently to provide a uniform suspension of the particles. Typically, the amount of particles in solution will be between about 0.5 mg per milliliter of solution and 5 mg per milliliter of solution. Sonication is a preferred method for providing a uniform suspension of the coated particles in solution.

The suspension of coated particles and gene segments must be within certain parameters for segment attachment and self assembly to occur. The temperature of the particle solution should be between 1°C to 45°C. The gene segments or antisense fragments may be bound to the coated particles in distilled water. The oxygen tension of the solution is preferably less than 10% in a solution sparged initially by helium and then gassed with helium, nitrogen and carbon dioxide. The pH of the solution is preferably slightly acidic (relative to blood), with a value, preferably, of between 6.8 to 7.2. An exemplary solution for dispersion of the coated microparticles and for DNA attachment is an aqueous solution containing: 0.0360 milligrams MgSO_4 per liter, 0.0609 milligrams $\text{MgCl}_{2.6}\text{H}_2\text{O}$, 0.0441 milligram $\text{CaCl}_{2.2}\text{H}_2\text{O}$, 22.823 grams K_2HPO_4 , 13.609 grams KH_2PO_4 , 7.455 grams KCl , and 4.101 gram sodium acetate. The pH of this solution is adjusted to 6.8.

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The coated particle cores with the attached gene segment or antisense fragment can be separated from the ionic growth medium and stored for further use. The coated particles may be stored by any of the conventional methods typically used for storing gene segment or antisense fragments. For example, the coated particles may be freeze dried or stored as a suspension in a compatible solution. When used in gene therapy, the particles coated with a targeting layer as described below, are injected or otherwise administered to the individual according to conventional procedures. Any pharmaceutically acceptable carrier solution or other compound may be used in administering the DNA/RNA coated particles to the individual. When used *in vitro*, the DNA/RNA coated particles are suspended in solution and used in the same manner as other gene therapy compounds. The same is true for use of antisense coated particles. The same protocol and procedures well known for gene therapy to introduce genes into cells both *in vivo* and *in vitro* may be used wherein the DNA/RNA/antisense-particle constructs of the present invention are substituted for other gene therapy compounds.

Targeting of the coated particle and attached gene support or antisense fragment is accomplished by coating the particles with a phospholipid membrane complex which includes ligands that are reactive with receptors on particular cells. Exemplary target ligands include HIV coat proteins (gp160, 41, 120) corona virus coat proteins, EBV coat proteins (gp350). Any membrane bound ligand/receptor may be used. These ligands are attached to the particle complex in the same manner as attachment of the transfection agents discussed above.

The lipids used to coat the biodegradable nanocrystalline particle and bound transfection agent are the same lipids commonly used to form liposomes. Suitable lipids include phospholipids such as phosphatidylcholine, cholesterol and phosphatidylserine. The lipid layer is applied to the nanocrystalline core particle and bound to a biologically active agent in the same

manner as the other coatings are applied, i.e. by adsorption onto the surface.

The core particle and bound agent do not need to be totally covered with a lipid layer. Preferably, the amount of lipid used to coat the particle will be sufficient to coat the entire particle. The combined layer of lipid and targeting ligand provide for targeting of the core particle and attached gene segments to the corresponding cell receptor.

The preferred transfection nanoparticles are self-assembling complexes of nanometer sized particles, typically on the order 100 nm, that carry an inner layer of transfecting DNA or RNA and an outer layer of targeting molecules. Functionally, the targeting molecules, usually referred to as ligands, impart tissue specificity in the same way a virus finds its host, i.e., the ligands promote the association of the transfection nanoparticles to a cell surface by binding to cell surface receptor molecules.

Construction of the transfection nanoparticles is a simple process and which occurs spontaneously without apparent covalent modification. In one exemplary synthesis, nanoparticle dispersions of tricalcium phosphate (TCP) are prepared from isochoric opposing streams of 0.750 M Calcium Chloride and 0.25 M monobasic Sodium Phosphate. The resulting precipitant is sonicated at 175 Watts at room temperature for 30 minutes and washed in volumes of 20 mM pH 6.80 phosphate buffer before being mixed with Cesium Chloride purified transfecting DNA, RNA or antisense. The DNA, RNA or antisense is then left to adsorb to the particulate surfaces at room temperature under mild agitation. After the transfection agent attachment step is completed, membrane specific ligands, typically prepared from the envelopes of retroviruses, are added to the dispersion and allowed to adsorb overnight at 4.0°C in a stir cell.

The ligand receptor complex can be chosen so as to be unique for the targeted tissue since tissues can be differentiated by their component cells, cell surface receptors, and complementary ligands. Once the interac-

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tion takes place, transfection can proceed through a range of cellular uptake mechanisms, resulting in the dissolution of the DNA (RNA/anti-sense) away from the particle complexes, recombination, and expression in a target cell. Introducing DNA, RNA or antisense by this method allows for the alteration of the phenotype of specific cells in a targeted tissue. It will occur because of the conformational stability of the ligands employed, the integration locus of the transfecting DNA/RNA/antisense, and the expression ability of transfecting DNA (RNA/antisense) in the targeted tissue.

Ligand preparation is as varied as their source. They can be produced by recombinant means or derived from their natural source. Preferably, viral envelope ligands are extracted from viruses, such as Human Immunodeficiency Virus, Epstein-Barr Virus, and murine ecotropic viral strains. In general, the viral envelopes are extracted in accordance with known procedures and then combined with phospholipids in a phosphate buffer. The solution of viral envelopes and phospholipids are then added to the suspension of DNA/RNA coated nanoparticles. The viral envelopes and phospholipids are absorbed onto the nanoparticles to form a targeting membrane.

Typical preparations of transfection nanoparticles yield in the neighborhood of a tenth of a microgram of DNA (and RNA) per microliter of dispersion as gauged by spectrophotometric determinations over time. If higher concentrations are required, the DNA (and RNA) is premixed with the substrate solutions and is allowed to slowly coprecipitate with the core material at a pH of 6.5. The particulate size is controlled by the time wise addition and surface adsorption of membrane ligands, and by the removal of substrate by ultrafiltration dialysis. Independent of the synthetic route chosen, administration of the transfection nanoparticles is accomplished by both enteral and parenteral routes. The doses are the same as those used in gene therapy.

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The nanoparticle-DNA/RNA constructs of the present invention may be made without the targeting layer when receptor mediated targeting is not required. For example, the constructs may be prepared without the targeting layer when calcium channel uptake of DNA/RNA or other non-cell receptor uptake is desired. The small size of the constructs allows them to evade the reticular endothelial system, thereby increasing the circulation time and transfection efficiency.

The following non-limiting examples describe certain aspects of the present invention in greater detail.

Example 1. Preparation of nanocrystalline tin oxide microparticles:

1.5 to 2.0 mg of ultrafine (nanocrystalline) metal powder was placed in a 1.7 ml screw-cap microcentrifuge with 1.5 mls of double distilled water (ddH₂O). The ddH₂O was filtered through a rinsed 0.45 micron filter-sterilizing unit or acrodisc (Gelman Scientific). The metal powder was tin oxide with a mean diameter (by photon correlation spectroscopy) of 140 nm. The mixture was vortexed for 30 seconds and placed into a water sonicating bath overnight. The sonication bath temperature was stabilized at 60°C. After a 24-hour sonication, the samples were vortexed once more for 30 seconds with the resulting dispersion clarified by microcentrifugation at approximately 16,000 rpm for 15 seconds. The analysis of particle size was carried out on a Coulter N4MD sub-micron particle analyzer.

The coating was applied to the tin oxide particles by suspending the particles in a stock solution of cellobiose. The cellobiose stock solution was a 292 mM solution made by dissolving 1.000 gram of cellobiose in 9.00 mls of ddH₂O. Solution was accomplished at approximately 70°C in order to promote quick dissolution. The resulting cellobiose solution was filter sterilized through a rinsed 0.45 micron filter with the final volume being adjusted to 10.00 ml.

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Sufficient cellobiose stock solution was added to 150 microliters of ultrafine tin oxide dispersion so that the final concentration of the tin oxide was 1.00 percent (w/v) or 29.2 mM. A typical volume for preparation was 2.0 mls which was mixed four or five times by the action of a micro-pipetor. After mixing, the dispersion was allowed to equilibrate for two hours. Demonstration of successful coating of the particles was provided by measuring the mobility of the particles (coated and uncoated) on a Coulter DELSA 440 doppler energy light scatter analyzer. The coated tin oxide particles exhibited a relatively low mobility compared to the non-coated tin oxide particles. Measurements were also taken at various dilute salt concentrations to ensure that the observations with respect to mobility were not artifactual. The tests demonstrate that the particles were coated with the cellobiose.

The coated particles are then used to attach antigenic proteins, peptides or pharmacological agents to prepare bioreactive particles.

Example 2. Preparation of nanocrystalline ruthenium oxide particles:

The same procedure was carried out in accordance with Example 1, except that ruthenium oxide microparticles were substituted for the tin oxide particles. The ruthenium oxide particles were obtained from Vacuum Metallurgical Company (Japan).

Example 3. Preparation of the nanocrystalline silicon dioxide and tin oxide particles:

Nanocrystalline silicon dioxide was acquired commercially from Advanced Refractory Technologies, Inc. (Buffalo, N.Y.) and tin oxide was acquired commercially from Vacuum Metallurgical Co. (Japan). The tin oxide particles were also prepared by reactive evaporations of tin in an argon-oxygen mixture and collected on cooled substrates. Nanocrystalline tin oxide was also synthesized by D.C. reactive Magnetron sputtering (inverted cathode). A 3" diameter target of high purity tin was sputtered

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in a high pressure gas mixture of argon and oxygen. The ultrafine particles formed in the gas phase were collected on copper tubes cooled to 77°K with flowing liquid nitrogen. All materials were characterized by X-ray diffraction crystallography, transmission electron microscopy, photon correlation spectroscopy, and Doppler electrophoretic light scatter analysis. X-ray diffraction samples were prepared by mounting the powder on a glass slide using double-sized Scotch tape. CuK α radiation was used on a Norelco diffractometer. The spectrum obtained was compared with ASTM standard data of tin oxide. (Powder Diffraction File, Card #21-1250. Joint Committee on Powder Diffraction Standards, American Society for Testing and Materials, Philadelphia 1976.) The specimens for (TEM) were collected on a standard 3 mm diameter carbon coated copper mesh by dipping into a dispersion of the (UFP's) in 22-propanol. The samples were examined on a JEOL-STEM 100 CX at an acceleration voltage of 60-80 KV.

To create working dispersions of these metal oxides, 1.5 to 3.0 mg of metal oxide powder was added to 1.5 ml double distilled H₂O in a dust-free screw top microcentrifuge tube (Sarsted) and vortexed for 30 seconds. The mixture was then sonicated for 16 to 24 hours followed by a second 30 seconds vortex. The submicron fraction was then isolated by pelleting macroparticulates by microcentrifugation 16,000 $\times g$ for 15 seconds. Approximately 1.3 ml of supernatant was then removed and placed in another dust-free screw top microcentrifuge tube. A sample was prepared for photon correlation spectroscopy (Coulter N4MD) and Doppler electrophoretic light scattering (Coulter delsa 440) analysis by removing 50 to 100 μ l of the dispersion and placing it in a polystyrene cuvette and diluting it to a final volume of 1.00 ml with ddH₂O. The stability of the dispersion was determined by sequential measurements over a 24-hour period and was found to be stable. The stability of the dispersion with respect to progressive salinity of the solvent (increasing conductivity) was

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similarly determined. The stability increased with progressive salinity of the solvent.

1.00 ml of the dispersion was combined and stirred with 8.00 ml of ddH₂O and 1.00 ml of 29.2 mM cellobiose stock in a 15.0 ml capacity ultrafiltration stir cell (Spectra) which has been fitted with a pre-rinsed 5x10⁵ molecular weight cutoff type F membrane (Spectra). The sample was then left to stir for 15 minutes. After stirring, the excess cellobiose was removed by flushing through the cell chamber 250 ml of ddH₂O by the action of a peristaltic pump at a rate that does not exceed 10.0 ml/min. After washing, the filtrate was concentrated by the means of pressurized N₂ gas to approximately 1.0 ml. Character was established by the removal of 500 ul of the treated dispersion by N4MD analysis. The mean dispersion diameter was re-established at this step. The stability of the coated dispersion was determined by sequential measurements over a 24-hour period. The stability of the coated dispersion with respect to progressive salinity of the solvent (increasing conductivity) was similarly determined.

The resulting coated nanocrystalline particles are suitable for attachment of various proteins, peptides and pharmaceutical agents.

Example 4. Preparation of Tricalcium Phosphate (TCP) Nanocrystalline Particles Coated With P5P:

1. Using two 60 cc syringes and a T-Luer lock, inject 50 mls of 0.75 m CaCl₂ and 50 mls of 0.25 m Na₂HPO₄ into a 120 ml pharmaceutical bottle in the cup sonicator. Sonicate for 30 minutes at room temperature to form suspension of TCP particles.

2. Spin the TCP preparation down in the centrifuge using the bucket rotor at 3000 rpm for 15 minutes to remove unreacted components.

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3. Resuspend the TCP particles in 50 mls of HPLC grade water and mix well. Spin down at 3000 rpm for 15 minutes. Repeat step 3, three times (3x).

5 4. Add 1.0 ml of 100 mg/ml pyridoxal-5-phosphate (P5P) and incubate for 30 minutes on a rocker arm at room temperature.

5. Lyophilize overnight.

10 6. Resuspend the P5P-TCP preparation in 50 mls of 0.1 n sodium hydroxide. Mix well. Spin down at 3000 rpm for 20 minutes. This removes the excess P5P. (This step may not have to be completed with all carbohydrates. Centrifugation and subsequent washing steps may be adequate.)

15 7. Resuspend in 50 mls of PBS and spin down at 3000 rpm for 15 minutes. Repeat step 8 three times (3x). This removes the sodium hydroxide.

20 8. Resuspend pellet in 50 mls of HPLC grade water and spin down at 3000 rpm for 15 minutes. Repeat step 9 three times (3x). This removes the PBS.

25 9. Resuspend the pellet in 4.0 mls of HPLC water and 1.0 ml of 100 mM of sodium citrate to pH 7.2.

10. Sonicate for 15 minutes at room temperature to form suspension of particles which is ready for attachment of biochemically active agent.

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Example 5. Preparation of TCP Nanocrystalline Particles Coated with Cellobiose:

The same procedure as described in Example 1 is followed except that cellobiose is substituted for P5P. The cellobiose coating is applied to the particles by suspending the particles in a stock solution of cellobiose. The cellobiose stock solution is a 292 mM solution made by dissolving 1.000 gram of cellobiose in 9.00 mls of ddH₂O. Solution is accomplished at approximately 70°C in order to promote quick dissolution. The resulting cellobiose solution is filter sterilized through a rinsed 0.45 micron filter with the final volume being adjusted to 10.00 ml.

Sufficient cellobiose stock solution is added to 150 microliters of the ultrafine biodegradable particle dispersion so that the final concentration of the particle is 1.00 percent (w/v) or 29.2 mM. A typical volume for preparation is 2.0 mls which is mixed four or five times by the action of a micropipetor. After mixing, the dispersion is allowed to equilibrate for two hours. Demonstration of successful coating of the particles is provided by measuring the mobility of the particles (coated and uncoated) on a Coulter DELSA 440 doppler energy light scatter analyzer. The coated particles exhibit a relatively low mobility compared to the non-coated particles. Measurements are also taken at various dilute salt concentrations to ensure that the observations with respect to mobility are not artifactual.

The coated particles are then used to attach antigenic proteins, peptides or pharmacological agents to prepare bioreactive particles.

Example 6. Preparation of Nanoparticles with Cellobiose or P5P Coatings: The tin oxide, ruthenium oxide and silicon dioxide nanoparticles prepared in Example 1-3 are coated with cellobiose or P5P in the same manner as TCP.

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Example 7. Preparing Meticulously Clean Biodegradable Nanoparticles:

1. Prepare 6 clean sonication tubes with 500 mg of biodegradable particles per tube.
2. In fume hood, fill tubes with HCl (10 N) approx. 8 ml/tube.
3. Sonicate for 30 min. (full power [175 watts]/25°C); three tubes per sonication treatment.
4. Centrifuge 30 min. at 2000 rpm.
5. Decant the acidic supernatant (in the fume hood), fill the tubes with HPLC grade water and then vortex.
6. Sonicate for 30 min [above conditions] and centrifuge for 30 [centrifuging is complete if the supernatant is clear].
7. Decant the supernatant, and fill the tubes with HPLC grade water and vortex.
8. Repeat steps 7 and 8 two more times.
9. Decant the preparation into a clean glass [pyrex] baking dish.
10. Anneal at 210°C overnight.
11. Remove the dried biodegradable crystals by gentle scraping with a clean unpainted spatula and transfer into 6 clean glass sonicating tubes.

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12. Repeat steps 3 through 8.
13. Prepare a 10 kD (NMWL) 150 ml ultrafiltration cell, empty the contents only one[no more than 500 mg per filtration run] of the tubes into the cell, and wash 500 ml of HPLC grade water through the cell under a N₂ pressure head of 20 psi (regulator pressure gauge reading).
14. After washing, adjust the preparation volume to 100.0 ml by using the appropriate volume markings on the side of the cell.
15. Take a concentration measurement by removing 1.0 ml of the preparation from the cell and lyophilizing it down in a pre-weighed 1.7 ml Eppendorf tube. After lyophilization, take a mass measurement of the tube with its contents and subtract it away from the mass of the empty tube. This provides the initial density of the preparation. Preferably, the concentration or density of the particles in the solution is about 10 mg/ml. If the initial density is lower than 10 mg/ml, then the solution should be further concentrated in the ultrafiltration cell.

Example 8. Coating Meticulously Clean Biodegradable Nanoparticles with a Molecular Stabilizing Film (Cellobiose):

Incubation/Lyophilization.

1. Sonicate the meticulously clean biodegradable particles (aqueous dispersion) prepared in Example 7 for 30 minutes at 25°C at full power [175 Watts].

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2. Then as quickly as possible, exchange suspending medium from water (stock) to a solution of 500 mM cellobiose using either a bench top microcentrifuge (30 seconds, full speed of 14,000 RPM) for small volumes or for larger volumes a floor models centrifuge (model 21K, in 50 ml centrifuge tubes, 8,000 RPM for a maximum of 2 minutes). Suspend the pelleted particles with 500 mM cellobiose, sonicate to aid dispersion (approximately 5 minutes at 25°C at full power [175 Watts]) and finally set the mixture on a rocking plate overnight in a cold room [4°C].
3. The next day portion out the mixture into appropriately sized vessels for overnight lyophilization.
4. Leave the tubes capped with a layer of parafilm around the cap and place them in a freezer until the washing step.
5. Reconstitute the particle/cellobiose in a suitable buffer depending on the application. Suitable buffers are low ionic strength buffered phosphate (PRB), water, or bicarbonate. Reconstitution in the buffer is accomplished by vortexing and a 5 minute sonication [175 Watts/25°C].
6. Wash by repeated centrifugation (using either a bench top microcentrifuge [30 seconds, full speed of 14,000 RPM] for small volumes or for large volumes a floor model centrifuge [model 21K, in 50 ml centrifuge tubes, 8,000 RPM for a maximum of 2 minutes]) and resuspension into the buffer.

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7. Take a concentration measurement by removing 1 ml of the suspension dehydrating it in a lyophilizer in a pre-weighed 1.7 ml Eppendorf tube, and massing.

5 8. Calculate the final volume necessary to bring the concentration to 1 mg/ml. Add enough buffer to bring the concentration of the particle/cellobiose preparation to 1 mg/ml.

Example 9. Preparing Meticulously Clean Particles of Brushite:

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Reagents. 0.75 M CaCl_2 : 55.13g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ is dissolved with HPLC grade water to 0.500L in a volumetric flask. Filter sterilize with 0.2 μm sterile filtration unit and place in a sterile 500 ml culture medium flask. Store at room temperature.

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0.25 M Na_2HPO_4 : 17.75g of anhydrous Na_2HPO_4 is dissolved with HPLC grade water to 0.500 L in a volumetric flask. Filter sterilize with 0.2 μm sterile filtration unit and place in a sterile 500 ml culture medium flask. Also store at room temperature.

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Brushite synthesis. About a half hour before synthesis, prepare the sonicator by cooling down the cup horn. This is accomplished by adjusting the low temperature thermostat on the water condenser to 4°C and dialing a setting of "4" on the peristaltic circulator. Once the 4°C mark is reached, prepare 50.0 ml of 0.75 M CaCl_2 and 50.0 ml of 0.25 M $\text{Na}_2\text{H}_2\text{PO}_4$ and load into 50 ml syringes. The syringes are then to be connected to a 3-way luer lock connector so that they are set in diametric opposition — allowing the remaining luer port to be free to dispel product. Once the mixing apparatus is set up, place a sterile 120 ml sonicating flask in the cup horn and slowly power up the sonicator to

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over the sonicating flask. Expel syringe contents into the flask as rapidly and evenly as possible so as to empty each syringe roughly at the same time. Then quickly secure a polypropylene liner over the sonicating flask and let sonicate for an additional 15 minutes.

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Brushite washing. Roughly divide the preparation into two 50 ml blue top polypropylene tubes and pellet at 2000 rpm for 10 minutes (room temperature). Reconstitute by vortexing each pellet with sterile HPLC grade water to 50 ml (or tube capacity) and pellet at 2000 rpm for 10 minutes. Repeat this wash 3 more times and reconstitute the last pellets to 50.0 ml. Transfer the dispersion to a sterile 120 ml sonicating flask with polypropylene liner. Place the flask in a previously cooled sonicator cup horn at 1°C. Sonicate at 100% power for 60 minutes.

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Example 10. Coating Meticulously Clean Particles of Brushite with a Molecular Stabilizing Film of Pyridoxyl-5-Pyrophosphate:

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Brushite/Pyroxidal 5 phosphate (vitamine B6). Pellet 100 ml of the dispersion prepared in Example 10 so that the entire contents can be transferred to a 50 ml conical tube. Adjust the tube volume to 40.0 ml. Then transfer the contents in 10 ml aliquots to four 15 ml conical tubes. Dissolve 1000 mg of Pyroxidal-5-phosphate with 800 μ l of 10 N NaOH and adjust with water to 10 mls. Filter sterilize this clear yellow solution with a .2 μ m acrodisc and add 2.5 ml aliquots to each of the previously prepared 4 brushite tubes. Vortex each tube a few seconds to make certain that the contents are well dispersed. Lyophilize overnight [approx. 16 hrs] at the low drying rate setting. The next morning resuspend in 50 ml aliquots of sterile HPLC grade water five more times. Pellet once more and transfer the pellets to four 15 ml conical tubes and adjust the final preparation volume with water to 40.0 ml.

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Example 11. Coating Meticulously Clean Particles of Brushite With a Molecular Stabilizing Film of Citrate:

Brushite/citrate. Pellet the 100 ml of the dispersion prepared in Example 13 so that entire contents can be transferred to a 50 ml conical tube. Adjust the tube volume to 40.0 ml. Then transfer the contents in 10 ml aliquots to four 15 ml conical tubes. Add 10 ml of 100 mM citrate to each of the 15 ml conicals and nutate for 30 minutes at room temperature. Lyophilize overnight [approx. 16 hrs] at the low drying rate setting. The next morning resuspend in 50 ml aliquots of sterile HPLC grade water five more times. Pellet once more and transfer the pellets to four 15 ml conical tubes and adjust the final preparation volume with water to 40.0 ml.

Example 12. Preparation, isolation and surface adsorption of human serum transferrin proteins: Nanocrystalline tin oxide was synthesized by D.C. reactive Magnetron sputtering (inverted cathode). A 3" diameter target of high purity tin was sputtered in a high pressure gas mixture of argon and oxygen. The ultra-fine particles formed in the gas phase were collected on copper tubes cooled to 77°K with flowing liquid nitrogen. All materials were characterized by x-ray diffraction crystallography, selected area electron diffraction, transmission electron microscopy, photon correlation spectroscopy, and energy dispersive x-ray spectroscopy. X-ray diffraction samples were prepared by mounting the powder on a glass slide using double-sized Scotch tape. CuK(*alpha*) radiation was used on a Norelco diffractometer. The spectrum obtained was compared with ASTM standard data of tin oxide. The specimens for transmission electron microscopy and selected area diffraction were collected on a standard 3 mm diameter carbon coated copper mesh by dipping into a dispersion of the nanocrystalline materials in 2-propanol. The samples were examined

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on a JEOL-STEM 100 CX at an acceleration voltage of 60-80 KeV. The 2-propanol suspension of particles was also characterized by photon correlation spectroscopy at 22.5°C, 600 s run time on a Coulter N4MD. Energy dispersive x-ray spectroscopy was performed on a JEOL JSM-T330A scanning electron microscope using Kevex quantex V software.

To create working dispersions of these metal oxides for the synthesis of compositions in accordance with the present invention, 0.5 mg of metal oxide powder was added to 1.0 ml of a 29.2 mM cellobiose-phosphate buffered saline solution in a dust free screw top glass vial and sonicated for 20 minutes at 22.5-35°C. The submicron fraction was then isolated by pelleting macroparticulates by microcentrifugation at 16,000xg for 30 seconds. Approximately 900 μ l of supernatant was then removed and placed in a dust free screw top microcentrifuge tube. An aliquot was removed for photon correlation spectroscopy (Coulter N4MD) and Doppler electrophoretic light scattering (Coulter DELSA 440) analysis. Aliquots were also removed for characterizing the stability of the coated dispersion over time and with respect to progressive salinity of the solvent (increasing conductivity).

To adsorb protein to the cellobiose coated metal oxide nanocrystalline cores, the core sample was diluted to 10.0 ml with Ca^{++} and Mg^{++} free phosphate buffered saline (Gibco). Forty (40.0) μ g of purified human serum transferrin (4 μ g/ μ l) (Gibco), whose antigenicity was verified by ELISA, was then added to a 10 ml stir cell (Spectra). The sample was then left to stir slowly for 30 minutes, taking great care not to allow foaming. After the addition period, 15 ml of Ca^{++} and Mg^{++} free phosphate buffered saline (Gibco) was then washed through the cell under a 2 psi nitrogen gas pressure head. After washing, the sample was again concentrated to 1.00 ml under N_2 and a 500 μ l sample was removed for analysis by photon correlation spectroscopy, Doppler electrophoretic light scatter and transmission electron microscopy as detailed below.

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Conformational integrity was assessed by measuring the retained antigenicity of the bound protein. To the sample cell, 50.0 μ l of rabbit polyclonal anti-human transferrin antibody (Dako), whose antigenicity was confirmed by ELISA, was added to the concentrated 1.0 ml reaction product at 37.5°C with gentle stirring. After a 30 minute incubation period, 15 ml of Ca^{++} and Mg^{++} free phosphate buffered saline (Gibco) was then washed through the cell under a 2 psi nitrogen gas pressure head and the reaction volume was again reduced to 1.0 ml.

A 200 μ l aliquot of blocking agent, 1% w/v bovine serum albumin in divalent free saline, was added followed by a 10 minute equilibration period. The secondary antibody, 30 nm gold conjugated goat anti-rabbit polyclonal IgG (Zymed), was then added and the reaction mixture was allowed to incubate for 30 minutes. A sample was removed, chopped on a transmission electron microscopy grid, and vacuum dried. The mixture was again washed with 15 ml of divalent free saline under a nitrogen pressure head and then fixed with glutaraldehyde. One ml of 3% solid bovine collagen (Collagen Corp.) was then added to the mixtures and the composite was ultracentrifuged at $10^6 \times g$ for 30 minutes yielding a pellet that was then routinely processed as a biological specimen for transmission electron microscopy. Ten nm thick sections were viewed on a Zeiss transmission electron microscopy. Control samples were prepared as above without the cellobiose intermediate bonding layer.

Transmission electron micrographs showed that the D.C. magnetron sputtered tin oxide was composed of individual particles measuring 20-25 nm in diameter which aggregated into clusters measuring 80 to 120 nm in diameter. By photon correlation spectroscopy, these same particles when dispersed in distilled water produced agglomerates measuring 154 ± 55 nm. The tin oxide particles were fully crystalline as characterized by electron and x-ray diffraction. Energy dispersive x-ray spectroscopy showed no other elements present as impurities.

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By Doppler electrophoretic light scatter analysis, tin oxide exhibited a mean mobility of $2.177 \pm 0.215 \mu\text{m-cm/V-s}$ in aqueous solutions ranging from 10.8 to 20.3 μM NaCl. Following cellobiose surface coating in a 1% solution, tin oxide exhibited a mean mobility of $1.544 \pm 0.241 \mu\text{m-cm/V-s}$ in aqueous solutions ranging from 0.0 to 21.0 μM NaCl. The oxide agglomerated in salt concentrations of greater than 40.0 μM and in solutions of increasing cellobiose concentration.

Following transferring binding, the crude tin oxide/cellobiose/protein conjugates measured $350 \pm 84 \text{ nm}$ by photon correlation spectroscopy and transmission electron microscopy. Vacuum dried dropped samples with low concentration gold antibody measured 35-50 nm. Without the cellobiose bonding layer, vacuum dried sections measured 400 to $> 1000 \text{ nm}$. Occasional antibody bonding was noted. Following high concentration immunogold labeling and filtering, the thin section cellobiose treated specimens measured 50-100 nm. Positive gold binding was identified in approximately 20% of the appropriately coated samples whereas negative controls (prepared as above but lacking the primary rabbit antibody) exhibited approximately 1% nonspecific binding.

As can be seen from the above examples, the biological activity of protein absorbed to the surface of carbohydrate-treated nanocrystalline metal oxide particles is preserved.

Example 13. Preparation and Characterization of Epstein-Barr Virus Decoys: Nanocrystalline tin oxide particles were synthesized by D.C. reactive Magnetron sputtering as previously described in Example 1.

Elutriated sucrose gradient purified Epstein-Barr virus (EBV) acquired from the B95-8 cell line were purchased from Advanced Biotechnologies, Inc., Columbia MD. Each viral aliquot contained approximately 5.00×10^{10} virus particles/ml suspended in 10mM TRIS-150mM NaCl pH 7.5 buffer (approximately 0.94 mg/ml protein). The virions were solubilized 0.75%

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(v/v) Triton X100 and then ultracentrifuged at 150,000xg for 60 minutes to pellet the DNA core using a modification of the method described by Wells. (Wells A, Koide N, Klein G: Two large virion envelope glycoproteins mediate EBV binding to receptor-positive cells. J Virology 1982; 41:286-297.) Following dialysis, the supernatant EBV extract was characterized by both SDS-PAGE (denatured) [Biorad Mini Gel II, 4-20% gradient gel, 200V x 45 minutes and stained with silver] and size exclusion HPLC (non-denatured) [Waters 620 system with a WISP autoinjector and 720 photodiode array detector, 0.5 ml/minute over a Waters SW300 GFC column using a 100mM NaCl/20mM TRIS pH 9.4 gradient mobile phase].

Control (non-EBV) proteins were extracted from aliquots of Lambda phage virus [Pharmacia, Milwaukee WI] using the same methods as described above.

Aliquots of the tin oxide powder weighing approximately 1.5 mg were initially suspended in 3.0 ml of 29.2 mM cellobiose solution in a dust free glass vial by liberal vortexing [Vortex Genie, Scientific Industries, Bohemia, NY]. The resultant brownish cloudy suspension was then sonified at 175 W for 10.0 minutes at a frequency of approximately 20 kHz at 25°C [Branson 2" Cup Horn, Branson Ultrasonics Corp., Danbury CT]. The dispersion was clarified by microcentrifugation at 16,000xg for 15 seconds. The remaining pellet was then discarded in favor of the supernatant. Unadsorbed cellobiose was removed by ultrafiltration against 20 mls of 25 mM phosphate reaction buffer (pH 7.40 25mM $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^{1-}$) in a 10 kD nominal molecular weight filtered stir cell [Pharmacia] under a 7.5 psi N_2 gas head at 37.5°C. Aliquots of the intermediate product were characterized by photon correlation spectroscopy and, following dialysis as described below, by doppler electrophoretic light scatter analysis.

The process of viral protein adsorption was initiated by the removal of the mild triton surfactant from 250 μl aliquots of EBV extract by ultrafiltration against 25 mls of phosphate reaction buffer at 4°C in a 10

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kD nominal molecular weight stir cell and then adjusted to a concentration of 1.0 $\mu\text{g}/\mu\text{l}$ or approximately 1.0 ml final volume. Then 500 μl of the triton free EBV extract was quickly added to a MD nominal molecular weight stir cell with 2.0 ml of the surface treated tin oxide dispersion prewarmed to 37.5°C. The mixture was then slowly stirred while being incubated at 37.5°C for 2.0 hours. After incubation the unabsorbed EBV extract was removed by ultrafiltration against 25 mls of phosphate reaction buffer.

Control (non-EBV) decoys fabricated with lambda phage viral protein extracts were synthesized using the same process described above.

Intermediate components, the final assembled decoys, and whole Epstein-Barr virions were characterized by doppler electrophoretic light scatter analysis [DELSA 440, Coulter Electronics Inc., Hialeah, FL] to determine their electrophoretic mobility (surface charge) in a fluid phase. Nine phosphate buffer solutions having at 25°C pH's ranging between 4.59 and 9.06 and corresponding conductivities ranging between 2.290 and 4.720 mS/cm were prepared. Aliquots of raw tin oxide, surface modified cellobiose covered tin oxide, synthesized EBV decoy, and whole EBV were dialyzed against each of the nine solutions and the mobilities of the particulates in dispersion were then measured at field strengths of 4.0, 5.5, 5.5, and 8.0 mA respectively. The mobility values acquired simultaneously by the 4 angled detectors of the instrument were averaged and the means of 3 measurements per dispersion were recorded.

The synthesized EBV decoys and control decoys were characterized by immunoagglutination photon correlation spectroscopy to determine the antibody reactivity of their surfaces. Positive reactivity was assessed by incubating the EBV decoy for 60 minutes at 37.5°C with a cocktail of anti-EBV murine monoclonal antibodies (1 μg each of anti-EBV-VCA, anti-EBV EA-R, anti-EBV MA, and anti EBV EA-D) in 15% lactose, 0.9% NaCl, 10 mM HEPES buffer, and 0.2% NaN₃ [DuPont, Wilmington, DE]).

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Background reactivity was assessed by incubating the EBV decoy with irrelevant murine IgG₁. Specificity was assessed by reacting the lambda phage decoy with monoclonal anti-EBV murine antibodies. Agglutination was measured by photon correlation spectroscopy at a 90° angle [N4MD, Coulter].

Antibody affinity intensity was assessed by immunogold transmission electron microscopy using the particulates and antibodies listed above and then adding secondary anti-murine 30 nm gold-labeled antibodies (Faulk W, Taylor G. Immunocolloid method for electron microscopy, *Immunochemistry* 8:1081-1083, 1971).

Labeling of the EBV decoy (positive reaction) was accomplished by incubating a 20 μ l mixture of murine monoclonals (1 μ g anti-EBV-VCA and 1 μ g anti-EBV EA-R in 15% lactose, 0.9% NaCl, 10 mM HEPES buffer, and 0.2% NaN₃ [DuPont]) with a fresh 0.5 ml sample of EBV decoy at 37.5°C for 30 minutes in a 300 kD nominal molecular weight stir cell. Unbound antibody was then removed by ultrafiltration against 20 mls of phosphate reaction buffer under a 5.0 psi N₂ pressure head. After washing, 50 μ l of goat anti-murine antibody covalently fused to 30 nm gold spheres (10⁶ particles/ml[Zymed Laboratories, San Francisco, CA]) were incubated with 200 μ ls of the labeled particles in a 1M nominal molecular weight stir cell at 37.5°C for 30 minutes. Unbound secondary antibody was removed by ultrafiltration against 10 mls of phosphate reaction buffer.

Labeling of the EBV decoy (negative reaction) was accomplished by incubating 2.5 μ l of murine polyclonal nonspecific IgG1 (1 μ g/ μ l in 15 mM NaCl pH 7.4 [Sigma Chemical Corp., St. Louis, MO]) with a fresh 0.5 ml sample of EBV decoy as described above followed by the same washing and gold-labeling steps. Labeling of the lambda phage control decoy (negative reaction) was accomplished by incubating a 20 μ l mixture of murine monoclonal anti-EBV antibodies with the lambda phage virus coated decoy using the same procedure detailed above.

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Immunolabeled particles were prepared for electron microscopy in two ways. A direct immersion technique where a carbon coated copper viewing grid [Ted Pella Inc., Redding, CA] was submersed into sample for approximately 5 seconds and then fixed in 5% glutaraldehyde for 1 minute, was used for all reactions as a fast screening technique. A more involved method adding glutaraldehyde directly to the reaction solution, then pelleting the product at 16,000xg for 5 minutes into 0.5 ml soft agar preparation (0.7% agarose [Sea Kem, Temecula, CA] in H₂O). Then the resultant agar plugs were embedded in plastic and sectioned into 0.1 μ m sheets for viewing.

Analysis of both the positive and negative controls was performed by examining pelleted samples of the labeled reaction products by transmission electron microscopy. The relative intensity of antibody binding was determined by counting the number of tin oxide based particles observed to have bound gold spheres (% positive) and then noting the number of gold spheres bound to a given particle (intensity, number/event).

The ultrafine tin oxide particles measured 20-25 nm in diameter and formed aggregates measuring 80 to 120 nm in diameter by transmission electron microscopy. By photon correlation spectroscopy, these same particles when dispersed in distilled water produced agglomerates measuring 154 ± 55 nm. The tin oxide particles were fully crystalline as characterized by electron and x-ray diffraction. Energy dispersive x-ray spectroscopy showed no other elements present as impurities.

Characterization of the EBV proteins by SDS-PAGE showed two distinct protein bands. The first, existing as a dimer suggesting variable glycosylation, exhibited a molecular weight of approximately 350 kd which is consistent with the predominant envelope glycoprotein of EBV. The second exhibited a molecular weight of approximately 67 kd consistent with serum albumin which apparently adsorbs avidly to the viral surface.

HPLC confirmed the presence of two distinct bands that exhibited spectrophotometric absorption maxima at 280 nm consistent with proteins. The predominant peak had a chromatographic retention time of 10.30 minutes and could be suppressed 90% by monoclonal anti VCA. The second and relatively minor peak exhibited a chromatographic retention time of 15.75 minutes similar to bovine serum albumin standards.

The previously described Doppler electrophoretic mobility studies conducted between the pH range of 4.5 to 9.0 demonstrated 3 distinct patterns. First, both the decoy and native EB virus retained virtually identical mobilities of approximately $-1.4 \mu\text{m-cm/V-s}$ throughout the pH range. Second, untreated tin oxide exhibited a mobility of approximately $-1.0 \mu\text{m-cm/V-s}$ at a pH of 4.5 which then rose rapidly to $-3.0 \mu\text{m-cm/V-s}$ at pH values of 5.0 and higher. Third, surface modified tin oxide treated with cellobiose retained a mobility of approximately $-1.5 \mu\text{m-cm/V-s}$ until it increased rapidly to $-2.5 \mu\text{m-cm/V-s}$ at a pH of 7.5.

The previously described photon correlation spectroscopy showed that native EBV measured approximately $102 \pm 32 \text{ nm}$ and the synthesized EBV decoy measured approximately $154 \pm 52 \text{ nm}$. Synthesized EBV decoy, when reacted with the monoclonal anti-EBV cocktail, agglutinated to form $1534 \pm 394 \text{ nm}$ masses. Synthesized EBV decoy, when reacted with non-specific mouse IgG, only increased slightly in size with agglutination diameters of $230 \pm 76 \text{ nm}$. Lambda phage decoy, when reacted with the monoclonal anti-EBV cocktail, only increased slightly in size with agglutination diameters of $170 \pm 35 \text{ nm}$.

The previously described transmission electron microscopy of anti-EBV antibody labeled EBV decoy particles revealed a positive gold staining frequency of $23.51\% \pm 5.53$ with an average staining intensity of 7.41 gold labels per event. Examination of non-specific mouse IgG antibody labeled EBV decoy particles revealed a positive gold staining frequency of $5.53\% \pm 2.04$ with an average staining intensity of 1.00

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gold labels per event. Examination of anti-EBV antibody labeled lambda phage decoy particles revealed a positive gold staining frequency of 7.21% +/-1.26 with an average staining intensity of 1.06 gold labels per event.

5 Example 14: *In Vivo* Elicitation of Antibodies By Epstein-Barr Virus

10 Decoy: Four sensitization solutions were prepared and delivered once every other week by intramuscular injection in three 250 μ l aliquots to New Zealand rabbits aged approximately 8 weeks. The first four animals received approximately 10^9 whole EBV virions (approximately 32 μ g of gp350 estimated by integration of the spectrophotometric absorption curve at 280 nm against a 25 μ g bovine serum albumin standard) dispersed in phosphate reaction buffer per injection. The second four animals received 32 μ g per injection of isolated and purified gp350 using the same injection protocol. The third group received EBV viral decoys (Example 5) synthesized from a starting aliquot of 32 μ g of gp350 per injection. The last group received cellobiose coated in tin oxide dispersed in phosphate reaction buffer. Injections were free of adjuvant. Whole blood was removed using aseptic techniques via cardiac puncture 2 weeks following each of the three injections and the animals were terminated by cardiac puncture followed by lethal sedation at 6 weeks. Serum was extracted by microcentrifugation at 16 kg of whole blood for 1 minute and then stored frozen at -70°C pending analysis.

20 Immunospecific antibody against whole EBV virions (ABI) was assayed by ELISA. Approximately 10^9 virions/ml in phosphate reaction buffer were diluted 1:10 in coating buffer and then allowed to adsorb overnight at 4°C in polycarbonate assay plates (Falcon). Rabbit serum affinity for the bound EBV virions was determined by the colorimetric reaction of goat anti-rabbit IgG alkaline phosphatase (Sigma) developed with para-nitrophenyl phosphate. The concentration of immunospecific IgG were determined by comparison to a calibration curve using nonspecific

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rabbit IgG as the adsorbed antigen and by subtracting the baseline values recorded from the wells containing serum from the rabbits stimulated with tin oxide only.

Serum collected from the 4 rabbits sensitized with tin oxide showed
5 no increased anti-EBV activity over pre-immune serum at any of the three two week sampling intervals. The remaining 3 groups showed a progressive rise in the concentration of anti-EBV specific IgG over the 6 week period. Animals sensitized with purified EBV proteins alone showed a maximum of approximately 0.05 ug/ μ l anti-EBV IgG at six weeks. In
10 contrast, animals sensitized with either whole EBV or decoy EBV exhibited a statistically significant four fold greater response with approximately 0.20 μ g/ μ l of anti-EBV IgG at six weeks. The immunospecific responses to decoy EBV and whole EBV were virtually identical.

As is apparent from Examples 5 and 6, the synthesized EBV decoy
15 in accordance with the present invention possesses the same surface charge as native virus, is recognized specifically and avidly by monoclonal antibodies, and evokes immunospecific antibodies with the same effectiveness as whole virus. Using photon correlation spectroscopy, the number of particles that agglutinated in the three reaction conditions were
20 calculated from the measured diameters of the aggregates. These calculations indicate that monoclonal anti-EBV antibodies produce agglutinated masses consisting of an average 988.0 decoy EBV particles. Non-specific mouse IgG antibodies produce agglutinated masses consisting of an average 3.33 decoy EBV particles, while monoclonal anti-EBV antibodies
25 produce agglutinated masses consisting of an average 1.35 decoy control lambda phage particles. These measured results show that the measured agglutination potential of the EBV decoy in accordance with the present invention is almost three orders of magnitude greater than controls. The immunogold transmission electron microscopy shows that the gold labeled
30 antibody staining of anti-EBV labeled EBV decoys is 25 to 30 times greater

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than controls. The ELISA analysis of the immunospecificity of anti-EBV IgG elicited in the rabbits by the EBV decoy is similar to the response elicited by native virus and is 4 fold greater than the response elicited by isolated purified proteins. Examples 5 and 6 are summarized in Kossovsky, N. et al., Nanocrystalline Epstein-Barr Virus Decoys, Journal of Applied Biomaterial, Vol. 2, 251-259, (1991).

Example 15. Preparation of HIV Decoys: The following procedure was used to adsorb HIV membrane antigens onto diamond nanocrystalline particles to provide HIV decoys.

HIV Workup. 1.0 ml of HIV (TCID 50 titre which varied between $10^{5.75}$ to $10^{7.17}$ as determined by the producer Advanced Biotechnology, Inc.) was dialyzed into PBS by 100 KD ultrafiltration and frozen down to -70°C until needed. On injection day the viral stock was thawed on ice and diluted to 1:25 in PBS. 100 ul of this preparation was used for injection. 1.0 ml of HIV ($10^{5.75}$ transforming units per ml) [ABI] was added to 0.5 ml of envelope extraction buffer [1.0% of Triton X 100\0.25 mM DTT\ 10 mM Tris pH 7.4\1.0 mM MgCl] and was allowed to incubate for 1.0 hr at room temp. The extract was then ultracentrifuged at $100K \times g$ for 2.0 hrs [35krpm SW50.1 Beckman rotor] at 4.0°C to remove nucleocapsid. Removal of Triton X and envelope protein enrichment was accomplished by incubation with a 300 ul slurry of polystyrene micro beads [Spectra Gel D2] and subsequent 100 kD ultra filtration into PBS. For a 100 ul injection the extract volume was corrected to a 1.0 ml volume and diluted 1:25 in PBS or to a protein concentration of around 2.5 ug/100ul/injection volume. Protein quantization was conducted by HPLC. HPLC conditions were as follows: Waters GFC SW300/Mobile phase:300mM NaCl, 20mM phosphates pH 7.4/ one major peak with a retention time of around 8.9 minutes

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at a flow rate of 0.5 ml per min/integration was done against BSA standards.

Preparation of HIV Decoy. HIV extract was adjusted to 1.0 ml volume after being ultrafiltered against pH 7.40 20 mM phosphate buffer and was incubated with 1.0 ml of diamond particles which had been coated with 500 mM cellobiose at 4.0°C for 24 hours. The diamond particles had an average particle size on the order of 50 nm. After adsorption the decoy dispersion was prepared for injection by 300 kD ultrafiltration against PBS to remove unadsorbed protein and was adjusted to 1.0 ml with PBS and parceled out for ten 100 µl injections.

Immunological Activity of HIV Decoy. Rabbits, guinea pigs, and mice were injected with either live virus, protein extract, protein extract mixed with Freund's adjuvant, or the HIV decoy virus. Antibody titres against whole virus were measured by ELISA and characterized by western blotting. Cell mediated reactivity was assessed in the guinea pigs by dermal skin challenge with live virus follows by biopsy.

At physiological pH, the mean electrophoretic mobility and average dispersion diameter (50 nm) of these synthetic carriers closely mimicked that of their infectious counterparts. Vaccination of mouse, guinea pig, and rabbit with the HIV decoy elicited the production of antisera which exhibited specific binding to whole HIV preparation as measured by ELISA. The histological analysis of earprick sites for animal sensitized to decoy virus and whole virus showed similar (qualitative and quantitative) reactions which differed significantly from both Freund's-sensitized animals and purified protein-sensitized animals at 1, 2, 7 and 24 weeks. Binding specificity was confirmed by Western blots.

As shown in the above example, the HIV decoy of the present invention has a number of characteristics which are shared with native whole

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5 HIV virus. These characteristics include: size, surface charge, immuno-
recognition, ability to elicit comparable antibody titers, and the magnitude
and character of cellular response. These attributes show that the decoy
virus in accordance with the present invention can function effectively as
a vaccinating agent.

10 Methods of obtaining meticulously clean solid surfaces, either by
cleaning pre-formed solids or by generating clean solids and surfaces *de*
novo; methods for applying solutions containing carbohydrates, carbohy-
drate derivatives, and other macromolecules with carbohydrate-like
components characterized by the abundance of -OH (hydroxyl) side groups;
methods for lyophilization to yield molecular stabilizing surface films; and
methods for immobilizing (a) member(s) of a BRP are described in the
additional examples below.

15 Example 16. Preparing a Meticulously Clean Carbon Ceramic
(Diamond) Nanoparticles:

1. Prepare 6 clean sonication tubes with 500 mg of particles per tube.
- 20 2. In fume hood, fill tubes with HCl (10 N) approx. 8 ml/tube.
3. Sonicate for 30 min. (full power [175 watts]/25°C); three tubes per
sonication treatment.
- 25 4. Centrifuge 30 min. at 2000 rpm.
5. Decant the acidic supernatant (in the fume hood), fill the tubes with
HPLC grade water and then vortex.

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6. Sonicate for 30 min [above conditions] and centrifuge for 30 [centrifuging is complete if the supernatant is clear].
7. Decant the supernatant, and fill the tubes with HPLC grade water and vortex.
8. Repeat steps 7 and 8 two more times.
9. Decant the preparation into a clean glass [pyrex] baking dish.
10. Anneal at 210°C overnight.
11. Remove the dried diamond crystals by gentle scraping with a clean unpainted spatula and transfer into 6 clean glass sonicating tubes.
12. Repeat steps 3 through 8.
13. Prepare a 10 kD (NMWL) 150 ml ultrafiltration cell, empty the contents only one[no more than 500 mg per filtration run] of the tubes into the cell, and wash 500 ml of HPLC grade water through the cell under a N₂ pressure head of 20 psi (regulator pressure gauge reading).
14. After washing, adjust the preparation volume to 100.0 ml by using the appropriate volume markings on the side of the cell.
15. Take a concentration measurement by removing 1.0 ml of the preparation from the cell and lyophilizing it down in a pre-weighed 1.7 ml Eppendorf tube. After lyophilization, take a mass measurement of the tube with its contents and subtract it away from the mass of the

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empty tube. This provides the initial density of the preparation. Preferably, the concentration or density of the particles in the solution is about 10 mg/ml. If the initial density is lower than 10 mg/ml, then the solution should be further concentrated in the ultrafiltration cell.

Example 17. Coating Meticulously Clean Diamond Nanoparticles with a Molecular Stabilizing Film (Cellobiose):

Incubation/Lyophilization.

1. Sonicate the meticulously clean carbon (diamond) (aqueous dispersion) prepared in Example 16 for 30 minutes at 25°C at full power [175 Watts].
2. Then as quickly as possible, exchange suspending medium from water (stock) to a solution of 500 mM cellobiose using either a bench top microcentrifuge (30 seconds, full speed of 14,000 RPM) for small volumes or for larger volumes a floor models centrifuge (model 21K, in 50 ml centrifuge tubes, 8,000 RPM for a maximum of 2 minutes). Suspend the pelleted carbon with 500 mM cellobiose, sonicate to aid dispersion (approximately 5 minutes at 25°C at full power [175 Watts]) and finally set the mixture on a rocking plate overnight in a cold room [4°C].
3. The next day portion out the mixture into appropriately sized vessels for overnight lyophilization.
4. Leave the tubes capped with a layer of parafilm around the cap and place them in a freezer until the washing step.

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5. Reconstitute the carbon/cellobiose in a suitable buffer depending on the application. Suitable buffers are low ionic strength buffered phosphate (PRB), water, or bicarbonate. Reconstitution in the buffer is accomplished by vortexing and a 5 minute sonication [175 Watts/25°C].

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6. Wash by repeated centrifugation (using either a bench top micro-centrifuge [30 seconds, full speed of 14,000 RPM] for small volumes or for large volumes a floor model centrifuge [model 21K, in 50 ml centrifuge tubes, 8,000 RPM for a maximum of 2 minutes]) and resuspension into the buffer.

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7. Take a concentration measurement by removing 1 ml of the suspension dehydrating it in a lyophilizer in a pre-weighed 1.7 ml Eppendorf tube, and massing.

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8. Calculate the final volume necessary to bring the concentration to 1 mg/ml. Add enough buffer to bring the concentration of the carbon/cellobiose preparation to 1 mg/ml.

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Example 18. Preparing Meticulously Clean Carbon Particles:

1. 2 grams of GE carbon powder was mixed with 25 ml 30% hydrogen peroxide + 75 ml 36N sulfuric acid in a 250 ml Belco stir flask (designed for suspension cultures). The reaction is exothermic and produces caustic vapors. Therefore it is advised to follow these precautions: 1. work inside a fume hood; 2. do not completely seal the Belco jar screw tops allowing ventilation through the 2 arms of the jar. Stir moderately for 8 days.

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2. Pour the solution into 2 x 50 ml centrifuge tubes (approximately 40 ml each). Discard the last 15-20% of the solution, saving only the whiter material. Spin the carbon to a pellet using 8,000 RPM for 1 minute (room temperature). Suspend pellet with 20 mM phosphate buffer (7.4). Wash 3 times. At the third washing step, the centrifugation period may need to be extended to 5-10 minutes since carbon is less precipitous with increasing pH. After the final wash, suspend pellet into HPLC water and store at room temperature. The resulting particles had a mean size of 260 nm +/- 87 nm.

- Results: Mean Size 260 nm +/- 87 nm; STD analysis 282 nm (98%) and 35.2 nm (2%) dust (4%)

Example 19. Immobilizing a Member of a Biochemically Reactive Pair (BRP) to a Coated Meticulously Clean Solid Surface:

1. One ml of Epstein-Barr virus EBV [ABI] was added to 4.0 ml of envelope extraction buffer [1.0% of Triton X 100\0.25 mM dithiothreitol\10 mM Tris pH 7.4\1.0 mM MgCl] and was allowed to incubate 1 hour at room temperature. The extract was then ultra-centrifuged at 100K *g for 2.0 hrs [35 krpm SW50.1 Beckman rotor] at 4°C to remove nucleocapsid.
2. Pellet is discarded in favor of supernatant.
3. The supernatant is transferred to a 100 kD ultrafiltration unit, kept cold with circulating water at 4°C.
4. Begin continuous dialysis using a total of 200 ml fresh, sterile PRB (20 mM phosphate, pH 7.4). For a 100 ul injection the extract

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volume was corrected to a 1.0 ml volume and, immediately prior to injection, is diluted 1:25 in PBS or to a protein concentration of around 2.5 ug/100 ul/injection volume. Protein quantification can be done by HPLC [HPLC conditions are as follows: Waters GFC SW300/Mobile phase: 300 mM NaCl, 20 mM phosphates pH 7.4/ one major peak with a retention time of around 8.9 minutes at a flow rate of 0.5 ml per min./Integration was done against BSA standards].

5. After transferring the EBV extract to the 100 kd filter unit, add the carbon/cellobiose particles prepared in Example 10 to a final concentration of 1 mg/ml. Begin continuous dialysis using a total of 200 ml fresh, sterile PRB (20 mM phosphate, pH 7.4). If the bound HBV is being prepared for injection, adjust to a final volume of 1.0 ml and dilute to 1:25 with PBS for a 100 ul injection. For all other uses, the bound HBV is stored at 4°C in PRB.

Example 20. Immobilizing a Member of Biochemically Reactive Pair (BRP) to a Coated Meticulously Clean Solid Surface:

1. Murine Lymphotropic virus (MuLV) extraction: MuLV stock [ABI] diluted 1:5 (e.g. 1 ml stock virus diluted to a final volume of 5 ml with the dilutant) with Triton X-100 extraction buffer [1.0% of Triton X 100\0.25 mM dithiotreitol\10 mM Tris pH 7.4\1.0 mM MgCl] and was allowed to incubate overnight at 4°C. The extract was then ultracentrifuged at 100K*g for 2.0 hours [35k rpm SW50.1 Beckman rotor] at 4°C to remove the nucleocapsid.
2. Pellet is discarded in favor of supernatant.

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3. MuLV decoy synthesis: It is desirable to use aseptic technique throughout the synthesis. Setup the stir cell unit such that access to the reaction mixture is rigorously controlled. Transfer the MuLV extract to a 100kd filter unit of 10 ml volume and add the carbon/cellobiose cores to a final concentration of 1 mg/ml. Begin continuous dialysis using a total of 200 ml fresh, sterile PRB (20 mM phosphate, pH 7.4). If the decoy is being prepared for injection, adjust to a final volume of 1.0 ml and dilute to 1:25 with PBS for a 100 ul injection. For all other uses, the decoy is stored at 4°C in PRB.

Example 21. Preparing a Meticulously Clean Solid Surface of Calcium Phosphate Dihydrate (Brushite):

Reagents. 0.75 M CaCl_2 : 55.13g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ is dissolved with HPLC grade water to 0.500L in a volumetric flask. Filter sterilize with 0.2 um sterile filtration unit and place in a sterile 500 ml culture medium flask. Store at room temperature.

0.25 M Na_2HPO_4 : 17.75g of anhydrous Na_2HPO_4 is dissolved with HPLC grade water to 0.500 L in a volumetric flask. Filter sterilize with 0.2 um sterile filtration unit and place in a sterile 500 ml culture medium flask. Also store at room temperature.

Brushite synthesis. About a half hour before synthesis, prepare the sonicator by cooling down the cup horn. This is accomplished by adjusting the low temperature thermostat on the water condenser to 4°C and dialing a setting of "4" on the peristaltic circulator. Once the 4°C mark is reached, prepare 50.0 ml of 0.75 M CaCl_2 and 50.0 ml of 0.25 M $\text{Na}_2\text{H}_2\text{PO}_4$ and load into 50 ml syringes. The syringes are then to be connected to a 3-way luer lock connector so that they are set in diametric

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opposition — allowing the remaining luer port to be free to dispel product. Once the mixing apparatus is set up, place a sterile 120 ml sonicating flask in the cup horn and slowly power up the sonicator to 100% power. Position the mixing apparatus so that the free luer port is over the sonicating flask. Expel syringe contents into the flask as rapidly and evenly as possible so as to empty each syringe roughly at the same time. Then quickly secure a polypropylene liner over the sonicating flask and let sonicate for an additional 15 minutes.

Brushite washing. Roughly divide the prep into two 50 ml blue top polypropylene tubes and pellet at 2000 rpm for 10 minutes (room temperature). Reconstitute by vortexing each pellet with sterile HPLC grade water to 50 ml (or tube capacity) and pellet at 2000 rpm for 10 minutes. Repeat this wash 3 more times and reconstitute the last pellets to 50.0 ml. Transfer the dispersion to a sterile 120 ml sonicating flask with polypropylene liner. Place the flask in a previously cooled sonicator cup horn at 1°C. Sonicate at 100% power for 60 minutes.

Example 22. Coating a Meticulously Clean Solid Surface of Calcium Phosphate Dihydrate (Brushite) with a Molecular Stabilizing Film of Pyridoxyl-5-Pyrophosphate:

Brushite/Pyroxidal 5 phosphate (vitamine B6). Pellet 100 ml of the dispersion prepared in Example 13 so that the entire contents can be transferred to a 50 ml conical tube. Adjust the tube volume to 40.0 ml. Then transfer the contents in 10 ml aliquots to four 15 ml conical tubes. Dissolve 1000 mg of Pyroxidal-5-phosphate with 800 μ l of 10 N NaOH and adjust with water to 10 mls. Filter sterilize this clear yellow solution with a .2 μ m acrodisc and add 2.5 ml aliquots to each of the previously prepared 4 brushite tubes. Vortex each tube a few seconds to make certain

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that the contents are well dispersed. Lyophilize overnight [approx. 16 hrs] at the low drying rate setting. The next morning resuspend in 50 ml aliquots of sterile HPLC grade water five more times. Pellet once more and transfer the pellets to four 15 ml conical tubes and adjust the final preparation volume with water to 40.0 ml.

Example 23. Coating a Meticulously Clean Surface of Calcium Phosphate Dihydrate (Brushite) With a Molecular Stabilizing Film of Citrate:

Brushite/citrate. Pellet the 100 ml of the dispersion prepared in Example 13 so that entire contents can be transferred to a 50 ml conical tube. Adjust the tube volume to 40.0 ml. Then transfer the contents in 10 ml aliquots to four 15 ml conical tubes. Add 10 ml of 100 mM citrate to each of the 15 ml conicals and nutate for 30 minutes at room temperature. Lyophilize overnight [approx. 16 hrs] at the low drying rate setting. The next morning resuspend in 50 ml aliquots of sterile HPLC grade water five more times. Pellet once more and transfer the pellets to four 15 ml conical tubes and adjust the final preparation volume with water to 40.0 ml.

Example 24. Immobilizing Insulin on Brushite:

Insulin Addition. 100 units of insulin is added to each of the four 10 ml suspension prepared in Example 15 and then agitated on a nutator at 4°C:

1) Lyophilization: Two of the core preparations are lyophilized overnight on a Savant Speed Vac (SVC100) under the low drying rate setting for approximately 16 hours. The next morning the lyophilate is resuspended to 10 ml with HPLC grade sterile water. Three washes with water are performed by pelleting and resuspension. Activity is determined

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during each wash by removing successive 1.0 ml aliquots and measuring the adsorption of light at 272 nm. Once it is determined that there is no activity in the supernatant (carrier) the preparation will have about 4.0 units per ml for injection. A typical injection is 500 ul [2.0 units].

5

If desired, the Brushite particles with the insulin immobilized thereon can be encapsulated in phospholipid as follows:

10

After insulin lyophilization, bring each of the preparations up to 10.0 ml with a water dispersion of 10% phosphatidyl choline, 10% phosphatidyl serine, and 5% water soluble cholesterol (Sigma Biochemical). Allow the mixture to incubate overnight at 4°C on a rocker. The next morning extrude the mixture through a 19 gauge needle without promoting significant foaming. Then three washes with water are performed by pelleting and resuspension. Activity is determined during each wash by removing successive 1.0 ml aliquots and measuring the adsorption of light at 272 nm. Once it is determined that there is no activity in the supernatant (carrier) the preparation will have about 4 units per ml for injection. A typical injection is about 500 ul [2.0 units].

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In vivo experiments show that intravenous injections of insulin immobilized on the coated brushite particles exhibit the same physiologic activity (serum glucose suppression) as solution phase insulin. Without the coating, insulin loses all biological activity when immobilized on brushite.

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Example 25. Immobilizing Bovine Serum Albumin on Zinc-Selenide Coated with Cellobiose: This example describes the modification of the surface of an analytic device used for Fourier transform infrared spectroscopy. The high energy surface of a meticulously cleaned ZnSe specimen holder is modified with a stabilizing film of cellobiose. While this provides a unique test surface for protein conformation analysis, it may be used to improve the biocompatibility of materials as well.

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Sample Preparation. The surface film coating the ATR specimen holder was comprised of cellobiose, and the analyte was bovine serum albumin. A solution of 100 mM cellobiose (Sigma's D-(+) cellobiose, FW = 342.3 solid-form) was prepared in HPLC-grade water (Sigma). Solid bovine serum albumin (Sigma, Mol. Wt. 46000) was dissolved in phosphate buffered saline (Sigma, Dulbecco's Phosphate Buffer, pH = 7.2) to obtain a desired concentration of 4% (w/v). All solutions were used within 14 days of preparation, and were stored at 4°C between experiments.

ATR Sample Holder Preparation. Prior to layering a thin desiccated film of cellobiose onto a horizontal ZnSe-45° ATR specimen holder (Spectra-Tech model, Stamford, CT), the plate was thoroughly washed with a solution of 100 mM NaCl and 100 mM NaCHO₃, followed by HPLC-grade water and acetone. A film of cellobiose was adsorbed onto the clean surface of the crystal by evenly applying 400 ul of 100 mM cellobiose and lyophilizing for ten minutes without applied heat or rotation (Savant SVC 100 lyophilizer, Wesbury, NY). 100 ul of the 4% BSA solution was then added onto the cellobiose coating. Excess protein solution was removed by gentle aspiration, and both the specimen and FTIR chamber were purged with N₂ for 15 minutes.

The immobilized BSA was analyzed by ATR-FTIR and found to have the same conformation as unbound aqueous phase BSA in terms of the proportional distribution of secondary structure components.

Example 26. Immobilizing Angiotensin Converting Enzyme on a Carbon Ceramic Core: Angiotensin converting enzyme was immobilized on carbon nanocrystalline particles coated with the procedures described in the previous examples. The rate of substrate cleavage in the solution phase, i.e. formation of BRP's, was found to be increased by a factor of five over the rate of substrate cleavage observed in the solution phase for

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non-anchored enzyme. The rate of substrate cleavage for angiotensin converting enzyme bound directly to uncoated particles was only 2.5 times that of the non-anchored enzyme.

5 Example 27. Preparation and Surface Adsorption of Human Serum Transferrin Protein of Brushite Particles Coated With Cellobiose: Brushite nanocrystalline particles are prepared as set forth in Example 9.

10 To adsorb protein to the cellobiose coated nanocrystalline cores, the core sample is diluted to 10.0 ml with Ca^{++} and Mg^{++} free phosphate buffered saline (Gibco). Forty (40.0) μg of purified human serum transferrin (4 $\mu\text{g}/\mu\text{l}$) (Gibco), whose antigenicity is verified by ELISA, is then added to a 10 ml stir cell (Spectra). The sample is then left to stir slowly for 30 minutes, taking great care not to allow foaming. After the addition period, 15 ml of Ca^{++} and Mg^{++} free phosphate buffered saline (Gibco) is then washed through the cell under a 2 psi nitrogen gas pressure head. After washing, the sample is again concentrated to 1.00 ml under N_2 and a 500 μl sample is removed for analysis by photon correlation spectroscopy, Doppler electrophoretic light scatter and transmission electron microscopy as detailed below.

20 Conformational integrity is assessed by measuring the retained antigenicity of the bound protein. To the sample cell, 50.0 μl of rabbit polyclonal anti-human transferrin antibody (Dako), whose antigenicity is confirmed by ELISA, is added to the concentrated 1.0 ml reaction product at 37.5°C with gentle stirring. After a 30 minute incubation period, 15 ml of Ca^{++} and Mg^{++} free phosphate buffered saline (Gibco) is then washed through the cell under a 2 psi nitrogen gas pressure head and the reaction volume is again reduced to 1.0 ml.

25 A 200 μl aliquot of blocking agent, 1% w/v bovine serum albumin in divalent free saline, is added followed by a 10 minute equilibration period. The secondary antibody, 30 nm gold conjugated goat anti-rabbit

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polyclonal IgG (Zymed), is then added and the reaction mixture is allowed to incubate for 30 minutes. A sample is removed, chopped on a transmission electron microscopy grid, and vacuum dried. The mixture is again washed with 15 ml of divalent free saline under a nitrogen pressure head and then fixed with glutaraldehyde. One ml of 3% solid bovine collagen (Collagen Corp.) is then added to the mixtures and the composite is ultracentrifuged at $10^6 \times g$ for 30 minutes yielding a pellet that is then routinely processed as a biological specimen for transmission electron microscopy.

Following transferring binding, the Brushite/cellobiose/protein conjugates will measure about 150 nm. The biological activity of protein absorbed to the surface of carbohydrate-treated nanocrystalline TCP particles is preserved.

Example 28. Preparing Meticulously Clean Biodegradable Nanoparticles:

1. Prepare 6 clean sonication tubes with 500 mg of biodegradable particles per tube.
2. In fume hood, fill tubes with HCl (10 N) approx. 8 ml/tube.
3. Sonicate for 30 min. (full power [175 watts]/25°C); three tubes per sonication treatment.
4. Centrifuge 30 min. at 2000 rpm.
5. Decant the acidic supernatant (in the fume hood), fill the tubes with HPLC grade water and then vortex.

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6. Sonicate for 30 min [above conditions] and centrifuge for 30 [centrifuging is complete if the supernatant is clear].
7. Decant the supernatant, and fill the tubes with HPLC grade water and vortex.
8. Repeat steps 7 and 8 two more times.
9. Decant the preparation into a clean glass [pyrex] baking dish.
10. Anneal at 210°C overnight.
11. Remove the dried biodegradable crystals by gentle scraping with a clean unpainted spatula and transfer into 6 clean glass sonicating tubes.
12. Repeat steps 3 through 8.
13. Prepare a 10 kD (NMWL) 150 ml ultrafiltration cell, empty the contents only one[no more than 500 mg per filtration run] of the tubes into the cell, and wash 500 ml of HPLC grade water through the cell under a N₂ pressure head of 20 psi (regulator pressure gauge reading).
14. After washing, adjust the preparation volume to 100.0 ml by using the appropriate volume markings on the side of the cell.
15. Take a concentration measurement by removing 1.0 ml of the preparation from the cell and lyophilizing it down in a pre-weighed 1.7 ml Eppendorf tube. After lyophilization, take a mass measurement of

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the tube with its contents and subtract it away from the mass of the empty tube. This provides the initial density of the preparation. Preferably, the concentration or density of the particles in the solution is about 10 mg/ml. If the initial density is lower than 10 mg/ml, then the solution should be further concentrated in the ultrafiltration cell.

Example 29. Coating Meticulously Clean Biodegradable Nanoparticles with a Molecular Stabilizing Film (Cellobiose):

Incubation/Lyophilization.

1. Sonicate the meticulously clean biodegradable particles (aqueous dispersion) prepared in Example 7 for 30 minutes at 25°C at full power [175 Watts].
2. Then as quickly as possible, exchange suspending medium from water (stock) to a solution of 500 mM cellobiose using either a bench top microcentrifuge (30 seconds, full speed of 14,000 RPM) for small volumes or for larger volumes a floor models centrifuge (model 21K, in 50 ml centrifuge tubes, 8,000 RPM for a maximum of 2 minutes). Suspend the pelleted particles with 500 mM cellobiose, sonicate to aid dispersion (approximately 5 minutes at 25°C at full power [175 Watts]) and finally set the mixture on a rocking plate overnight in a cold room [4°C].
3. The next day portion out the mixture into appropriately sized vessels for overnight lyophilization.
4. Leave the tubes capped with a layer of parafilm around the cap and place them in a freezer until the washing step.

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5. Reconstitute the particle/cellobiose in a suitable buffer depending on the application. Suitable buffers are low ionic strength buffered phosphate (PRB), water, or bicarbonate. Reconstitution in the buffer is accomplished by vortexing and a 5 minute sonication [175 Watts/25°C].
5
6. Wash by repeated centrifugation (using either a bench top micro-centrifuge [30 seconds, full speed of 14,000 RPM] for small volumes or for large volumes a floor model centrifuge [model 21K, in 50 ml centrifuge tubes, 8,000 RPM for a maximum of 2 minutes]) and resuspension into the buffer.
10
7. Take a concentration measurement by removing 1 ml of the suspension dehydrating it in a lyophilizer in a pre-weighed 1.7 ml Eppendorf tube, and massing.
15
8. Calculate the final volume necessary to bring the concentration to 1 mg/ml. Add enough buffer to bring the concentration of the particle/cellobiose preparation to 1 mg/ml.
20

Example 30. Preparation of Cellobiose Coated Brushite Particle with Hemoglobin Bound Thereto: The following example demonstrates the preparation of Brushite particle having hemoglobin bound thereto in accordance with the present invention. The fabrication process involved coating ultrafine nanocrystalline Brushite particles with a glassy film of disaccharides and then physically adsorbing purified hemoglobin. The assembly was then coated with phospholipid.
25

One (1.00) g. of ultrafine particles prepared as in Example 28 was dispersed in 5.0 ml of 100 mM cellobiose (Sigma) solution with 175 watt sonication (Branson) for 10 minutes. The colloid was then incubated at
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4.0°C overnight in a 10 kD stir cell. The following day, this colloid was lyophilized for 24 hours and reconstituted in 1.0 ml of ddH₂O. Unabsorbed cellobiose was removed by 10 kD stir cell ultra filtration (UF) (Filtron) against 100 ml of 20 mM phosphate buffer (pH 7.4) (PRB) and corrected to 2.0 ml. (UF) (Filtron) against 100 ml of 20 mM phosphate buffer (pH 7.4) (PRB) and corrected to 2.0 ml.

Five hundred (500) mg of human hemoglobin type A₀ (Sigma) was dissolved in 5.0 ml PBS (pH 6.8) (Gibco) and then ultrafiltered against 150 ml PRB at 4.0 C in a 50 kD stir with 30 psi N₂. The filtrate was adjusted to 3.0 ml. The surface modified Brushite dispersion (2.0 mL) was then added to the 50 kD ultrafiltrate cell and allowed to incubate overnight with slow stirring (5 psi N₂). The filtrate was adjusted to 3.0 ml. The surface modified diamond dispersion (2.0 mL) was then added to the 50 kD ultrafiltrate cell and allowed to incubate overnight with slow stirring (5 psi N₂, 4.0°C). The next morning, 35 uL of phosphatidyl dipalmitoyl serine (10 mM of 6.0 mM NaOH) (Sigma), 50 uL of phosphatidyl dipalmitoyl choline [6.8 mM stock] Sigma, and 8.7 uL of cholesterol [3.9 mM stock] (Sigma) was stirred in and incubated for approximately 6.0 hours. The final product was again filtered in a 50 kD ultrafiltration cell over 30 psi N₂ against 20 ml PBS (pH 7.4, 4.0°C and adjusted to 5.0 ml for an estimated hemoglobin concentration of 10 g/dL.

Example 31. Preparation of P5P Coated Brushite Particle with Hemoglobin Bound Thereto: In this example, red blood cell surrogates are made in the same manner as Example 30 except that pyridoxal-5-phosphate is substituted for cellobiose as the oxygen carrier anchor coating.

50 mg of acid cleaned particles was dispersed by 175 watt sonication (Branson) for 10 minutes, mixed with 75.0 mg of pyridoxal-5-phosphate [Sigma] and adjusted to 10.0 ml with deionized water. The mixture

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was spun lyophilized overnight, washed with 4-10 ml aliquots of deionized water and reconstituted to 25 mg/ml in pH 7.40, 20 mM phosphate buffer.

4.0 ml [25 mg/ml] of nanocrystalline core particles were added to 1.0 ml recovered red blood lysate hemoglobin [26.10 g/dL]. The mixture was then slowly dialyzed into 100 ml of 0.5X PRB overnight at 4.0°C under a nitrogen head of 20 psi and through a 10 kD ultrafiltration cell. The next morning, 34 μ L of phosphatidyl serine [10 mM of 6.0 mM NaOH] (Sigma), 50 μ L of cholesterol [3.9 mM stock] (Sigma) was stirred in and incubated for approximately 6.0 hours. The final product was again filtered to remove free hemoglobin in a 50 kD ultrafiltration cell over 30 psi N₂ against 200 ml PBS (pH 7.4, 4.0°C) and adjusted to 1.0 - 2.5 ml or an estimated hemoglobin concentration of 10 g/dL.

The preceding procedure was repeated at different pyridoxal-5-phosphate concentrations. As a result, red blood cell surrogates were prepared wherein the nanocrystalline particles were coated in solutions containing 1 mM pyridoxal-5-phosphate and 30 mM pyridoxal-5-phosphate.

The red blood cell surrogates prepared in Examples 30 and 31 were analyzed for oxygen dissociation characteristics as well as size distribution, electrophoretic mobility and retained molecular conformation. The red blood cell surrogates coated with cellobiose (Example 30) exhibited an oxygen dissociation (P50) of about 26-30 mm Hg. The red blood cell surrogates having coatings of pyridoxal-5-phosphate should have oxygen dissociations (P50) of about 37 mm Hg. This compares well with the oxygen dissociation of whole human blood which is 31 mm Hg. In addition, hemoglobin-bound nanocrystalline particles were prepared in the same manner as Example 30 except that the coating of lipid was deleted. The oxygen-dissociation of the lipid-free hemoglobin-bound nanocrystalline particles was about 10 mm Hg.

The electrophoretic mobility of the red blood cell surrogates produced in Examples 30 and 31 were measured with Doppler electro-

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phoretic light scatter analysis (DELSA 440, Coulter Electronics, Inc., Hialeah, FL). The electrophoretic mobility was $-1.7 \text{ } \mu\text{m cm/Vs}$ at a pH of 7.4 PRGB BUFFER at 25°C.

5 The size distribution of the red blood cell surrogates produced in Examples 30 and 31 were measured by both photon correlation spectroscopy at a 90° angle in PRB buffer at 22.5°C (N4MD, Coulter) and by transmission microscopy (TEM, Zeiss 190). The red blood cell surrogates measured about 200 nanometers by photon correlation. For electron microscopy, a 10 microliter drop of particles in solution was placed on a
10 paraffin surface which included a carbon-stabilized FORMVAR GRID (Ted Pella, Inc., Redding, CA) which was floated on top of the drop. Due to the high surface charge of the TEM GRID, the red blood cell surrogates absorbed to the grid allowing excess solution to be removed by careful blotting. A similar method was then used to stain the particles with 2%
15 phosphotungstic acid. The stained grid was then dried and the red blood cell surrogates identified as having particle sizes in the range of 50-100 nanometers.

The conformational integrity of the red blood cell surrogates was verified by immunogold antibody affinity intensity. After being deposited
20 on one nanometer TEM copper grids, the protein bound particles were incubated for one hour at 27°C with polyclonal rabbit anti-human hemoglobin antibodies (Dako) and secondary goat anti-rabbit 30 nanometer gold-labeled antibodies (Zymed Laboratories, San Francisco, CA). The gold-labeled antibodies were observed to attach avidly to the hemoglobin present in the red
25 blood cell surrogates.

Example 32. Preparation of P5P Coated Brushite Particle with Taxol Bound Thereto: In this example, drug delivery vehicles are made in the same manner as Example 30 except that taxol is substituted for hemo-
30 globin.

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50 mg of cleaned particles was dispersed by 175 watt sonication (Branson) for 10 minutes, mixed with 75.0 mg of pyridoxal-5-phosphate [Sigma] and adjusted to 10.0 ml with deionized water. The mixture was spun lyophilized overnight, washed with 4-10 ml aliquots of deionized water and reconstituted to 25 mg/ml in pH 7.40, 20 mM phosphate buffer.

4.0 ml [25 mg/ml] of nanocrystalline core particles were added to 1.0 ml solubilized taxol (100 mg/10 ml dd H₂O. The mixture was then slowly dialyzed into 100 ml of 0.5X PRB overnight at 4.0°C under a nitrogen head of 20 psi and through a 10 kD ultrafiltration cell and then lyophilized. The next morning, 34 uL of phosphatidyl serine [10 mM of 6.0 mM NaOH] (Sigma), 50 uL of cholesterol [3.9 mM stock] (Sigma) was stirred in and incubated for approximately 6.0 hours. The final product was again filtered in a 50 kD ultrafiltration cell over 30 psi N₂ against 200 ml PBS (pH 7.4, 4.0°C).

Example 33: Attachment of Transfection Agents:

The following procedure is used to attach DNA or RNA segments to any of the coated particles described in the preceding Examples:

A dispersion of 40 mg/ml of coated nanoparticles in 20 mM pH 6.80 phosphate buffer is prepared. To this dispersion, 1.00 ug of cesium chloride purified DNA or RNA fragments are added and left to absorb at room temperature under mild agitation for approximately 16 hours. Specific DNA fragment used in this example is the human deaminase gene.

Example 34: Attachment of Targeting Ligands and Phospholipid Membrane: The procedure described in the example for isolating and attaching the targeting ligands and phospholipid membrane may be used for all of the particle/coating/DNA or RNA combinations set forth in the preceding examples.

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5 10^6 transforming units of virus is incubated with Triton extraction buffer (1.0% of Triton X 100/0.25 mM DTT\10 mM Tris pH 7.4\ 1.0 mM MgCl). Extract is then ultracentrifuged at $100K \times g$ for 2.0 hrs (35 rpm SW50.1 Beckman rotor) at $4.0^\circ C$ to remove nucleocapsid. Removal of
10 triton and envelope protein enrichment is accomplished by incubation with a 300 ul slurry of polystyrene micro beads (Spectra Gel D2) and subsequent 100 kD ultrafiltration into phosphate buffer 5mg/ml of phosphotidyl choline and 5mg/ml phosphotidyl serine. An alternative method of viral extraction is as follows. A mixture of phosphotidyl choline (5mg/ml) and
15 phosphotidyl serine (5mg/ml) in 20 mM pH 7.4 phosphate buffer is sonicated for 30 minutes at $4.0^\circ C$. 10^6 units per ml of virus is then added to 1.0 ml of the phospholipid mixture and left to sonicate at 5 sec cycles per minute for 30 minutes at $4.0^\circ C$. Nucleocapsid is removed by ultracentrifugation at 35krpm at $4.0^\circ C$. Intervening layers of carbohydrate may be
20 first adsorbed to the TCP-DNA/RNA complex prior to the addition of the ligand/membrane components.

Example 35:

20 A construct is composed of a brushite core with an adsorbed first layer consisting of human deaminase gene in an expression cassette with a albumin enhancer and limited terminal repeats for genomic integration. The construct includes an adsorbed second layer of cellobiose and an adsorbed third layer of membrane proteins from human low density lipids (LDL receptor ligands).

25 The entire contents of all references cited hereinabove are hereby incorporated by reference.

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Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

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CLAIMSWhat is Claimed is:

1. A composition of matter comprising:
an article having a surface consisting of a metal, metal alloy,
polymer, ceramic, glass or intermetallic;
a coating consisting essentially of a material which provides
a threshold surface energy to said surface which is sufficient to bind
biochemically reactive pairs without denaturing said pairs; and
at least one biochemically reactive pair bound to said coated
surface wherein said biochemically reactive pair bound to said coated
surface is not denatured.

2. A composition of matter according to claim 1 wherein said
article is a core particle having a diameter of less than about 1000
nanometers.

3. A composition of matter according to claim 1 wherein said
bioreactive pair is selected from the group consisting of ligand-receptor
pairs, enzyme-substrate pairs, drug-receptor pairs, catalyst-reactant pairs,
immunological pairs toxin-ligand pairs, absorbant-absorbate pairs and
adsorbent-adsorbate pairs.

4. A composition of matter according to claim 1 wherein said
bioreactive pair is selected from the group of ligand-receptor pairs
consisting of lectins and lectin binding sites, HDL and HDL receptor cellular
receptor site, hormones and hormone receptor sites, antibiotics and
ribosomal proteins.

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5. A composition of matter according to claim 1 wherein said bioreactive pair is selected from the group of immunological pair members.

6. A composition of matter according to claim 1 wherein said bioreactive pair is selected from the group of enzyme-substrate pairs consisting of lysozyme-chitin pairs, ribonuclease-RNA pairs, carboxypeptidase A-carboxyl terminal polypeptide pairs, serine, zinc, thiol and carboxyl proteases-protein pairs, NADH-Q reductase-NADH pairs, glutathione seductase-glutathione pairs and acetylcholinesterase-acetylcholine pairs.

7. A composition of matter according to claim 1 wherein said bioreactive pair is selected from the group of drug-receptor pairs consisting of epinephrine and adrenergic receptors, methadone and opiate receptors.

8. A composition of matter according to claim 1 wherein said bioreactive pair is selected from the group of toxin-ligand pairs consisting of strychnine and the glycine receptor, hemoglobin and carbon monoxide, and organophosphate compounds and acetylcholinesterase.

9. A composition of matter comprising:

a biodegradable core particle having a diameter of less than about 1000 nanometers;

a coating comprising a substance that provides a threshold surface energy to said core particle which is sufficient to bind biochemically active agents without denaturing said agents, said substance covering at least a part of the surface of said core particle; and

at least one biochemically active agent bound to said coated core particle wherein said biologically active agent bound to said coated core particle is not denatured.

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10. A composition of matter according to claim 9 wherein said core particle comprises a biodegradable ceramic or polymer.

11. A composition of matter according to claim 10 wherein said core particle comprises a biodegradable ceramic selected from the group consisting of brushite and tricalcium phosphate.

12. A composition of matter according to claim 9 wherein said composition further comprises an outer coating of a phospholipid which comprises target ligands.

13. A composition of matter according to claim 9 wherein said biochemically active agent is one or both members of a bioreactive pair.

14. A composition of matter according to claim 9 wherein said coating comprises citrate, cellobiose or pyridoxal-5-phosphate.

15. A composition of matter according to claim 9 wherein said biologically active agent is a viral protein fragment.

16. A composition of matter according to claim 12 wherein said biologically active agent is a drug.

17. A composition of matter according to claim 9 wherein said biochemically active agent is hemoglobin.

18. A composition of matter comprising:
a biodegradable core particle having a diameter of less than about 1000 nanometers;

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5 a coating comprising a substance that provides a threshold surface energy to said core particle which is sufficient to bind transfection agents without denaturing said agents, said substance covering at least a part of the surface of said core particle; and

10 at least one transfection active agent bound to said coated core particle wherein said transfection agent bound to said coated core particle is not denatured.

19. A composition of matter according to claim 18 wherein said core particle comprises a biodegradable ceramic or polymer.

20. A composition of matter according to claim 19 wherein said core particle comprises of brushite.

21. A composition of matter according to claim 18 which further includes a target coating covering at least a portion of the surface of said core particle.

22. A composition of matter according to claim 21 wherein said outer target coating comprises a phospholipid and targeting ligand.

23. A composition of matter according to claim 18 wherein said coating comprises cellobiose pyridoxal-5-phosphate or citrate.

24. A composition of matter according to claim 18 wherein said transfection agent is a DNA or RNA segment.

25. A composition of matter according to claim 18 wherein said transfection agent is an antisense fragment.

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26. A composition of matter according to claim 22 wherein said targeting ligand is a viral envelope.

27. A composition of matter according to claim 21 wherein said core particle comprises a biodegradable ceramic or polymer.

28. A composition of matter according to claim 21 wherein said core particle comprises of brushite.

29. A composition of matter according to claim 21 wherein said coating comprises cellobiose pyridoxal-5-phosphate or citrate.

30. A composition of matter according to claim 21 wherein said transfection agent is a DNA or RNA segment.

31. A composition of matter according to claim 21 wherein said transfection agent is an antisense fragment.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12515

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 9/54, 9/56

US CL : 424/490, 491, 493, 494, 498

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/490, 491, 493, 494, 498

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,501,726 (SCHRODER ET AL.) 26 February 1985, column 2, lines 27 through column 3.	1-2, 9, 18
Y	CA, A, 1 252 950 (GRIES ET AL.) 18 April 1989, page 8.	1-2, 9, 18

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 DECEMBER 1994

Date of mailing of the international search report

12 JAN 1995

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN		
Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	<p>PAUSER S ET AL: "Evaluation of efficient chemoembolization mixtures by magnetic resonance imaging therapy monitoring: an experimental study on the VX2 tumor in the rabbit liver."</p> <p>CANCER RESEARCH, (1996 APR 15) 56 (8) 1863-7. JOURNAL CODE: CNF. ISSN: 0008-5472, XP002106971 United States siehe das ganze Dokument</p> <p>---</p>	1-33
Y	<p>DE 43 41 478 A (MAX DELBRUECK CT FUER MOLEKULA) 8. Juni 1995 siehe Spalte 1, Zeile 39 - Spalte 2, Zeile 53</p> <p>---</p>	1-33
Y	<p>WO 95 12392 A (UNIV CALIFORNIA) 11. Mai 1995 siehe Seite 7, Zeile 16 - Seite 12, Zeile 4</p> <p>---</p>	1-33
A	<p>DATABASE MEDLINE FILE SERVER STN KARLSRUHE ZUSAMMENFASSUNG 90358589, TAKEKOSHI H: "Chemoembolization combined with hepatic arterial induction of endogenous TNF and anticancer agents for hepatocellular carcinoma--a case report." XP002106973 siehe Zusammenfassung & GAN TO KAGAKU RYOHO 'JAPANESE JOURNAL OF CANCER AND CHEMOTHERAPY!', (1990 AUG) 17 (8 PT 2) 1744-7. JOURNAL CODE: 6T8. ISSN: 0385-0684.,</p> <p>---</p>	
A	<p>EP 0 132 142 A (HITACHI CHEMICAL CO LTD) 23. Januar 1985</p> <p>---</p>	
P,Y	<p>RESZKA, R. C (1) ET AL: "A new drug carrier application system for an efficient targeted treatment of liver metastases."</p> <p>JOURNAL OF LIPOSOME RESEARCH, (FEB., 1998) VOL. 8, NO. 1, PP. 98. MEETING INFO.: SIXTH LIPOSOME RESEARCH DAYS CONFERENCE LES EMBIEZ, FRANCE MAY 28-31, 1998 ISSN: 0898-2104., XP002106972 siehe das ganze Dokument</p> <p>---</p>	1-33
P,X	<p>WO 98 47532 A (COCKBAIN JULIAN R M ;NYCOMED IMAGING AS (NO); GEN HOSPITAL CORP (U) 29. Oktober 1998 siehe Seite 5, Absatz 2 - Seite 17, Absatz 1; Ansprüche 22,29,30</p> <p>-----</p>	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

DE 98/03763

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9620698	A	11-07-1996	AU 4755696 A	24-07-1996
			CA 2207961 A	11-07-1996
			EP 0805678 A	12-11-1997
			JP 10511957 T	17-11-1998
DE 4341478	A	08-06-1995	WO 9618421 A	20-06-1996
			EP 0797456 A	01-10-1997
WO 9512392	A	11-05-1995	US 5460830 A	24-10-1995
			US 5462751 A	31-10-1995
			US 5460831 A	24-10-1995
			CA 2174244 A	11-05-1995
			EP 0726767 A	21-08-1996
			JP 9504790 T	13-05-1997
EP 0132142	A	23-01-1985	JP 60021764 A	04-02-1985
WO 9847532	A	29-10-1998	AU 7068698 A	13-11-1998

